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The Journal of Biochemistry, Vol. 42, No. 2, 1955

INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

IV. EFFECT OF SOME PROTEIN PERTURBATORS UPON THE CATALYTIC FUNCTION OF CATALASE

By MITSUO SUZUKI*, TARO TOMIMURA AND
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(Received for publication, December 15, 1954)

Studies on the structural modification of apoprotein molecule and on the catalytic activity of the corresponding enzyme can lead to information which may be valuable in the elucidation of the mechanism of enzyme action. Dealing with catalase, the present authors intended to study the effect of salicylate and benzoate upon its catalytic activity. The perturbating effect of these agents upon hemoglobin protein was firstly reported by Anson and Mirsky (*1*, *2*), then by Holden (*3*) as well as in our laboratory by Tsushima (*4*, *5*), Kikuchi and Tomimura (*6*). The findings of the latter workers seem to be of great importance for the elucidation of the mode of catalytic function of heme proteins in general with special reference to the role of their individual apoproteins. A slight and reversible degree of denaturation in hemoglobin molecule induced by these perturbators causes an awakening of the immanent catalytic activity of Fe which is otherwise kept latent in the native state of this heme protein. Beside its intrinsic catalatic activity, catalase posesses a peroxidatic activity as already reported by Keilin and Hartree (*7*), Hoppel and Porterfield (*8*), Anan (*9*) and Tauber (*10*, *11*), which seems to be of biological importance. It has been our special interest in the present study to see how far these relative specificities might be maintained when the protein part of catalase is structually affected by the perturbators.

EXPERIMENTALS

Materials—Equine liver catalase which had been purified by Shirakawa's method (*12*) was used.

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Method—Evaluation of enzyme activity : a) catalatic activity was expressed by the rate of O_2 evolution measured by the Warburg technique ; b) peroxidatic activity was measured spectrophotometrically by the purpurogallin method, using pyrogallol or pyrogallol plus ascorbate as the hydrogen donator.

pH of the reaction solutions was 7.4. Temperature about 26°. Density of the cuvette : 10 mm. Optical densities were measured by an electrospectrophotometer of Hitachi & Co.

RESULTS

I. Effect of Perturbators upon Catalase Activity

a) Under the Condition in which only the Catalatic Activity Prevails—Final concentration of catalase and H_2O_2 : $2.2 \times 10^{-10} M$ and $1.1 \times 10^{-2} M$, respectively. Temperature : 22°. pH : 6.8.

Into the reaction mixture under the above condition, benzoate or salicylate was further added. Rate of O_2 evolution decreased with increasing concentration of benzoate or salicylate. The results are shown in Fig.1 as A and B, in which the O_2 evolution in 4 minutes is given in percentage of that of the control experiments without perturbator. A is the value of benzoate experiment and B the salicylate experiment. Above $2.0 M$ of benzoate or $1.2 M$ of salicylate, O_2 evolution decreased significantly.

b) Under the Condition in which Both the Catalatic and the Peroxidatic Activities Are Simultaneously in Play—pH : 6.8. T=22°. Final concentrations of catalase and H_2O_2 : $2.4 \times 10^{-10} M$ and $5.9 \times 10^{-3} M$, respectively. When pyrogallol was added into this reaction mixture, O_2 evolution decreased proportionally as pyrogallol increased. When the concentration of pyrogallol was taken as 1/1000 of the H_2O_2 concentration (in moles), the amount of O_2 evolved within 10 minutes was as low as 39 per cent of the control experiment and purpurogallin was formed in the reaction mixture. The fact indicates that in this case the enzyme is active both as catalase and peroxidase. But, when to this system benzoate was further added, O_2 evolution increased in proportion to the increase of benzoate. The amount of O_2 evolved aproached to that of the control experiment without pyrogallol and benzoate. The recovery of the catalase activity was maximum at the benzoate concentration of $0.8 M$. Above this concentration of benzoate, the O_2 evolution decreased again and at the benzoate concentration of $1.8 M$, the O_2 evolution approached to that of the initial system. The recovery of catalatic activity by benzoate are shown in Fig. 1. The recovery of activity is expressed in percentage, taking the O_2 output of the system within 10 minutes without pyrogallol as 100 per cent and that with pyrogallol as 0 per cent. The percentage of the recovery by benzoate was plotted against the log concentration of benzoate. This was shown by the broken line in Fig.1, C. On the basis of these observation, it may be suggested that the effect of pyrogallol upon catalase is restricted by the addition of benzoate leading to the recovery of catalatic activity of the enzyme and that the further increase of

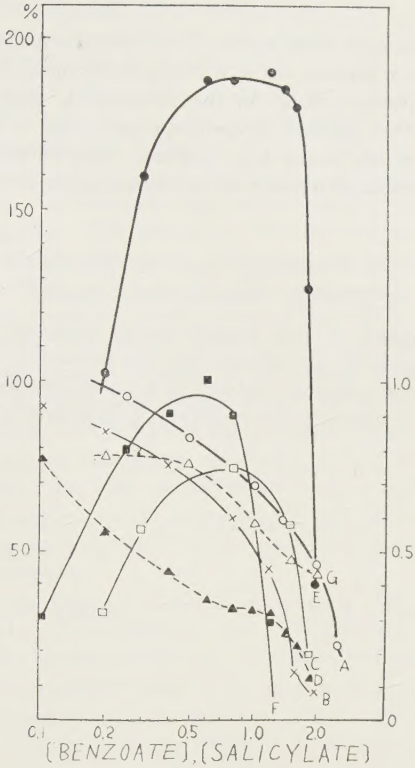

FIG. 1. Summarized effects of perturbators.

Abscissa : final log concentration of perturbators in *mole/lit.* Curves A (—○—○—) and B (—×—×—): effects of benzoate (A) and salicylate (B) upon catalase under the condition in which only catalatic activity is prevailing (manometric measurements). Curve C (—□—□—) : recovery effect of benzoate upon catalatic activity in the presence of pyrogallol (manometric measurement). Curue D (···▲···▲···) : inhibitory effect of salicylate upon peroxidatic activity with a lower concentration of H-donor (catalatic activity is simultaneously in play. Spectrophotometrical observation). Curves E (—●—●—) and F (—■—■—) : effect of benzoate (E) and salicylate (F) upon catalase under the condition in which peroxidatic activity is prevailing (photometrical). Curve G (···△···△···) : effect of benzoate upon absorption figure of catalase. Ordinate (scale on the right) : E_{380}/E_{415}.

benzoate may cause an inactivation of the enzyme.

c) *In Case where only Peroxidatic Activity Prevails*—When pyrogallol was sufficiently added to the system, the peroxidatic activity of the enzyme predominated so that the O_2 output could no more be observable. The following experiment was carried out to obtain the information whether any of the O_2 output can be proved by an addition of perturbator to the system under the above condition. The reaction system was composed of $2.4 \times 10^{-10} M$ catalase, $5.9 \times 10^{-3} M$ pyrogallol and $5.9 \times 10^{-3} M$ H_2O_2. Under this condition no O_2 output was observed. Also by addition of benzoate at $0.6 M$ as final concentration, no recovery of O_2 output could be observed in the reaction system.

II. Effect of Perturbators upon the Peroxidatic Activity of Catalase (Spectroscopical Studies)

a) Experimental Methods—It was manometrically confirmed using 10^{-10}—$10^{-7} M$ of catalase that there occurred no catalatic but only peroxidatic reaction, when pyrogallol was added into the system as H-donor in the concentration higher than that of H_2O_2. Firstly, it was examined as shown by Fig. 2 whether it is possible to determine the peroxidatic activity under these conditions by the purpurogallin coloration. Thus, pyrogallol was pipetted into the reaction vessel, covered with liquid paraffin, washed by carefully deoxygenated N_2 gas and catalase solution was then added to it. A slight coloration of purpurogallin occured already before the addition of H_2O_2. But, this is

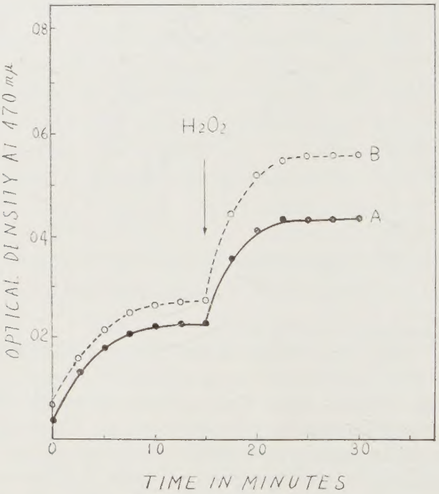

FIG. 2. Oxidatic and peroxidatic activity of catalase.
Zerro time : addition of catalase. Arrow : addition of H_2O_2. A : control. B : $0.6 M$ salicylate. reaction mixture : catalase 1.13×10^{-7}, H_2O_2 6×10^{-4}, pyrogallol $1.66 \times 10^{-2} M$; pH 7.4 ; T 26°.

due to the remaining oxygen which can be used up by catalase oxidatively. A similar result can be obtained in the experiment using crystalline catalase. The coloration induced by the oxidative activity of catalase ceased completely after 10 to 15 minutes. When H_2O_2 was then added after 15 minutes, the coloration by generated purpurogallin could be observed. Evidently this was produced by catalase peroxidatively.

The similar experiments were further carried out under the presence of salicylate. With regard to the extinction at 470 mμ, the coloration seemed to increase as shown in Fig. 2. (dotted line). But absorption figure of the reaction product was not identical with that of purpurogallin. The absorption at about 400 mμ has been decreased, while that at about 510 mμ increased. Thus, the reaction under the presence of salicylate proceeded to form a pigment other than purpurogallin and the extinction at 470 mμ increased with increasing concentration of perturbator. No isosbestic point was noticed in the mixture of purpurogallin and the new pigment. This makes it difficult to follow up the reaction precisely by the method applied as given in Fig. 2. The authors used thus for the present purpose an indirect method as follows. Into the reaction system of catalase, pyrogallol and H_2O_2, ascorbic acid was additionally mixed as a second H-donator. In this system, coloration of purpurogallin did not begin until the added ascorbate has been peroxidatively used up. The method has been firstly introduced by Tomimura for the estimation of peroxidatic activity of Cu ion (*13*). The relative activity of catalase could thus be easily compared by reading the time of the beginning of coloration.

b) *Experiments in the System with Lower Concentration of H-donator*—Under this condition catalase is active both catalatically and peroxidatically. The composition of the reaction system were devised as follows : $1.1\times10^{-8}M$ of catalase ; $2.8\times10^{-2}M$ of H_2O_2 ; $5.56\times10^{-3}M$ of pyrogallol ; $2.8\times10^{-4}M$ of ascorbate. pH 6.8 at 20°.

Under these conditions, owing to the relatively higher concentration of H_2O_2, both of the catalatic and peroxidatic reactions are active in the system. The length of time required for the formation of purpurogallin coloration were measured in this reaction system. The results are given in Fig. 3.

With increasing concentrations of salicylate added, the coloration of purpurogallin delayed. The relation between the concentration of salicylate and the peroxidatic activity of catalase is shown in Fig. 1, D (dotted line). The peroxidatic activity of catalase (ordinate) was expressed by the reciprocal of time length needed for the beginning coloration of purpurogallin, taking that of control experiment as 100 per cent. From the plotts, it is evident that the peroxidatic activity of catalase is inhibited by salicylate, the degree of inhibition being higher, the higher the concentrations of salicylate.

c) *Experiments with Sufficiently High Concentration of H-donor in the Reaction System*—The reaction system used was as follows : $1.07\times10^{-7}M$ of catalase ; $3.3\times10^{-3}M$ of pyrogallol ; $6.6\times10^{-4}M$ of ascorbate ; $1.3\times10^{-3}M$ of H_2O_2. pH 7.4 at 25°.

Under this condition of the system, no catalatic activity could be observable, but only peroxidatic activity of catalase. Now, sodium benzoate and sodium salicylate, respectively, was added into this reaction system and the time length needed for the biginning of purpurogallin coloration was measured.

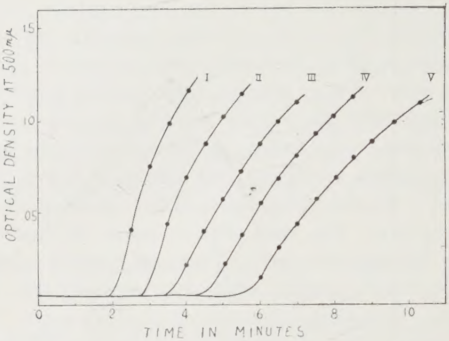

FIG. 3. Effect of salicylate upon purpurogallin formation in the presence of ascorbate.

I : without salicylate, II : 0.1 *M* salicylate, III : 0.2 *M* salicylate, IV : 0.4 *M* salicylate, V : 0.6 *M* salicylate.

The plotts are given in Fig. 1 (Curves E and F, respectively). In the figure, peroxidatic activity of catalase is expressed by the reciprocal of time length, taking that of control experiment as 100 per cent. As shown in Fig. 1 (E and F), the time needed for the biginning coloration of purpurogallin was considerably shortened in the presence of perturbators in lower concentrations. Under the effect of salicylate or benzoate in relatively lower concentration range, the rates of purpurogallin formation increased with increasing concentration of perturbators. When, however, the concentration of perturbators was increased above a certain limit, the rate decreased abruptly.

III. Absorption Change of Catalase as A Result of Perturbation

Catalase shows a strong absorption at 405 mμ (the Soret band). With increasing concentration of benzoate added to catalase solution, this Soret band shifted gradually toward longer wave length. In Fig. 4, the absorption changes of catalase solution after the addition of benzoate were plotted. The applied concentrations of benzoate were varied from 0.2 to 2.0 *M*.

Readings were made 60 minutes after the addition of perturbators. Concentration of catalase was $7.4 \times 10^{-7} M$. As shown in the figure an isosbestic point was clearly demonstrable at 415 mμ, so that the system may well be inferred to be composed of two components. Sodium benzoate shows an absorption at about 400 mμ. The illustrated figures are the absorptions corrected by subtracting the corresponding value of benzoate. Now $\varepsilon_{380} / \varepsilon_{415}$ was plotted against log concentrations of benzoate (Fig. 1, G). The plotts represent a sigmoid acurve. Thus the demonstrated alteration of the absorption figure may well be considered to indicate that some coordinative binding has been established at the 6th coordination position of protoheme Fe in the catalase molecule (refer to Tshushima's paper).

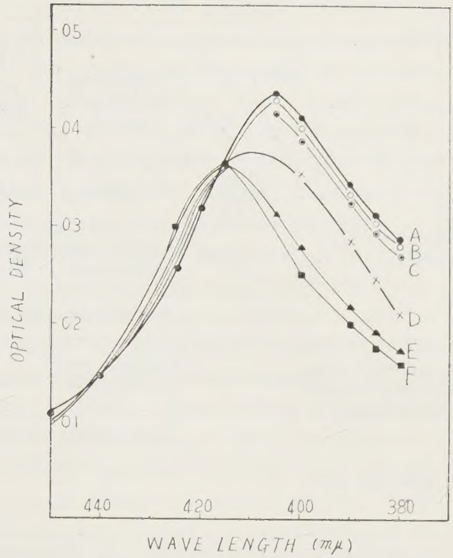

FIG. 4. Effect of benzoate upon the absorption figure of catalase (the Solet band)

A : without salicylate, B : 0.2 *M* salicylate, C : 0.5 *M* salicylate, D : 1.0 *M* salicylate, E : 1.5 *M* saliciylate, F : 2.0 *M* salicylate.

DISCUSSION

The main results of the present experiments are summarized in Table I and in Fig. 1. From these, some interesting facts may be pointed out.

Oxidase Activity of Catalase—It was revealed, as shown in Fig. 2, that catalase is significantly active as oxidase. The oxidative activity of catalase has hitherto been mostly overlooked.

Peroxidative Activity of Catalase—Peroxidatic activity of catalase has been demonstrated by several workers (*7*, *8*, *9*, *10*, *11*). It has repeatedly been shown that catalase can only be peroxidatively active when the concentration of H_2O_2 was low. For instance, as reported by Keilin and Hartree (*7*), ethanol could be peroxidatively oxidized by catalase in its concentration above $10^{-7}M$, only when H_2O_2 was added in concentrations less than 1/1000 of the concentration of catalase. Further, using benzidine as H-donor, Anan has proved that benzidine could

TABLE I

	H_2A* / H_2O_2	Prevailing catalytic activity	Effect of perturbator upon	
			Catalatic activity	Peroxidatic activity
From the measurements of O_2 output (manometrically)	without H_2A	Catalatic	Inhibition	
	1 : 1,000	Catalatic and peroxidatic	Recovery or promotion	Inoperable
	1 : 1	Peroxidatic	No recovery	Inoperable
From the readings of time length needed for the biginning coloration of purpurogallin (spectrophotometrically)	1 : 5	Catalatic and peroxidatic	Inoperable	Inhibition
	3 : 1	Peroxidatic	Inoperable	Promotion

* Pyrogallol or ascorbic acid.

more smoothly be oxidized peroxidatively by catalase only when the concentration of H_2O_2 in the system was kept as low as 1/1000 of heme concentration (*9*). From our results using pyrogallol or ascorbic acid, however, it could be shown that catalase can well be peroxidatively active even when more concentrated H_2O_2 was applied. We are rather of the opinion that catalase might be catalatic or peroxidatic only in response to the concentration ratio of H-doner to H_2O_2. Under the condition, in which the molar concentration of H- donor was held equal to that of H_2O_2, catalase was active almost completely as peroxidase. The peroxidatic activity of catalase may thus be controled by the proportional concentration of the components of the system namely of catalase, H_2O_2 and H-donator. Presumably, the most essential factor may be the oxidation reduction potential of the H-donor. The production of purpurogallin from pyrogallol through peroxidatic activity of catalase was reported also by Tauber (*11*) more recently.

Influence of Perturbators upon the Activity of Catalase—As indicated in Table I and Fig. 1, under the presence of perturbators, the protein part of catalase molecule underwent some modifications. The modification seems to proceed stepwise, through milder stage into more progressed stage.

Benzoate and salicylate affect catalase molecule in a similar manner. They differ only in their effective concentrations. Absorption change can distinctly be observable only at the sufficiently higher concentration range of the perturbators, thus in the more progressed state of structural modification. As far as the modification of catalase molecule remains in its milder stage, catalase shows more complicated activities. As indicated in Fig. 1, the activity is rather changeable according to the concentration ratio of H-donator and H_2O_2 in the reaction system. Without H-donator, activity of catalase decreased with increasing concentration of perturbators (Curves A or B in Fig. 1). But in the presence of H-donator in far lower concentration than that of H_2O_2 in the system, catalase is then both catalatically as well as peroxidatically active. When perturbator was added in this system, there occurred recovery of catalatic activity and decrease of peroxidatic activity (indicated as curve C in Fig. 1). However, when the concentration of H-donator in the system is higher than that of H_2O_2, the addition of perturbator effects a paramount promotion of peroxidatic activity of catalase.

The specificity of catalase depends undoubtedly upon the highly differentiated structure of the enzyme molecule. When this differentiated apoprotein structure is somewhat disintegrated by the effect of perturbation, for instance, the essential specificity of catalase may be lost in some way and instead there may appear the more generalized enzyme activity as peroxidase which is intrinsic to heme Fe. In the presence of H-donator in far lower concentrations relative to that of H_2O_2, however, the addition of perturbator to the system causes the decrease of peroxidatic and the recovery of catalatic activity. This findings seems to be incompatible with the above concept.

By addition of perturbators, both the catalatic and peroxidatic activity decreased abruptively. On such concentrations of perturbators, the absorption figure of catalase changes markedly, indicating a further binding of catalase heme with some of the heme-affined chemical groups in the molecule of catalase protein which has been opened out through the perturbation. It is well understandable that such a coordinatively saturated heme molecule shows any apreciable catalytic activity. Along with the absorption change caused by increasing concentration of perturbators, the absorption maximum of the Soret band shifted from 405 mμ to 415 mμ. In all of the compounds of catalase with H_2O_2, ROOH, HCN or HN_3, the absorption in this region generally shifts to 416～420 mμ.

Similar effects were more precisely studied by Tsushima recently with hemoglobin. Also the interrelation between the modifications of protein structure and that of the catalytical function of hemoglobin was experimentally studied by Tsushima, Kikuchi and Tomimura (*5, 6*). Our present studies dealing with catalase stands also on the same ground.

As to the possible objection that benzoate or salicylate inhibits the activity of catalase through anionic binding to catalase hemin, the authors would not come into agreement on the basis of the experimental facts established by Tsushima and Kikuchi. (*4, 5, 6*).

SUMMARY

The present experiment deals with the effects of perturbators such as sodium benzoate or salicylate upon the catalytic activity of catalase and the physical constitution of its protein molecule. The results can be summarized as follows:

1. The catalatic activity of catalase decreased with increasing concentration of perturbators added to the system. Under the presence of a small amount of pyrogallol in the reaction system, the enzyme is active both catalatically and peroxidatically. Under such a condition, catalatic activity is recovered by the addition of benzoate. When pyrogallol was added to the system in concentrations equivalent to H_2O_2, so that the enzyme can behave prevalently as peroxidase, no recovery of catalatic activity was observable by the addition of perturbators.

2. The peroxidatic activity of catalase was decreased by the addition of perturbator to the system only under the condition in which H-donor was added lower than H_2O_2, so that catalase can behave simultaneously as catalase as well as peroxidase. Under the condition in which, however, H-donor was added higher than H_2O_2, so that only peroxidatic activity prevails, the peroxidatic activity of catalase increased by the addition of lower concentration of perturbators.

3. Tle structural modification of catalase molecule by perturbators proceeds stepwise with the increasing concentration of perturbators. In higher concentration range of perturbators, an absorption change of catalase could actually be observed, indicating the occurrence of a more progressed modification of the molecule. In this state, the catalytic activity of catalase was greatly decreased.

4. It was proved that catalase shows an oxidative activity under certain experimental conditions.

We wish to express our cordial thanks to Prof. K. Kaziro, Dr. G. Kikuchi and Dr. R. Shukuya for their suggestions and kind advices during the course of this work.

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ENZYMATIC TREATMENT AND DYE BINDING ABILITY OF SERUM ALBUMIN

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(Received for publication, December 17, 1954)

Since Klotz *et al.* (*1*) reported the equilibrium dialysis method for the study of the binding of organic dye anions by bovine serum albumin, a great number of investigations of these kinds (*2*, *3*) have been published for various combinations of dyes and proteins.

With the spectrophotometric method described by Shimao (*4*) in this journal, the authors (*5*, *6*, *7*), performed studies on the binding of various dye ions by serum proteins (albumin and γ-globulin), and from their results discussed the difference in surface configurations between these native proteins and denatured ones.

Although the Klotz's method is applicapable in the case of proteins whose molecular weight is fairly large, it is difficult to use this method for the determination of dye binding ability of proteins which was hydrolyzed chemically or enzymatically. In this respect determinations are possible with Shimao's method regardless of molecular weight of proteins, and the results obtained by this method, therefore, will supply a new information of relation between extent of hydrolysis and dye binding ability, and moreover of difference in this relations due to the sorts or the specificities of proteolytic enzymes.

In this article the authors tried to study how the methyl orange binding ability of serum albumin was changed by peptic, tryptic or papaic digestion of the protein.

EXPERIMENTAL

Materials

Crystalline bovine serum albumin was prepared by Kekwick's method (*8*).

As a dyestuff, methyl orange, Merck's preparation was used.

As proteolytic enzymes, pepsin and trypsin, the preaparations of Nutritional and Biochemical Cooper. and Mochida Co. were used ; papain was obtained as a crude preparation from papaia.

Methods

Procedure of Proteolysis—To 1 g./dl. solution of bovine serum albumin whose pH has been adjusted properly, each enzyme was added, final concentration of trypsin or papain being 0.1 g./dl. and that of pepsin 5 mg./dl. This protein-enzyme system was incubated at 37±0.5° and the aliquot was pipetted out from it at various time intervals and offered to each determination.

Determination of Dye Binding Ability—Spectrophotometric determination of the bound dyes was carried out according to the Shimao's method (*4*) by the use of Beckman's spectrophotometer (Model Du). pH of the solutions offered to this determination was 6.7 in all cases, regardless of the pH at which proteolysis was performed.

Determination of Arginine Content in the Protein—It was carried out according to the Sakaguchi's method (*9*).

pH, Viscosity and Nitrogen Content in the Solution—They were determined by Beckman's glass electrode, Ostwald's viscosimeter at 25±0.1° and Parnas' apparatus respectively.

RESULTS

1) Comparison of the Rate of Survival of Protein Based on Non Protein Nitrogen Determination with That Based on Dye Bindind Ability Determination—As illustrated in the following figures, by the enzymic hydrolysis of serum albumin methyl orange binding ability expressed by E′-value of the total system (*cf.* reference (*4*)) decreased with the splitting time. Accordingly, referring to the Fig. 1, R_D calculated by the following Eq. (1).

(1) $E'(t, P_o) = E'(o, p_1)$

first variable in the bracket : time of hydrolysis

second variable in the bracket : initial protein concentration.

$$R_D = (p_1 / p_o) \times 100$$

can be taken as the percentage of unhydrolyzed protein in the solution, and R_D thus calculated was compared with R_N, where 100-R_N was the percentage hydrolysis obtained by the usual non protein nitrogen determination after trichloroacetic acid (TCAA) precipitation of the hydrolyzate at 0.15 *N* TCAA. E′-values were determined at pH 6.7 in the presence of *M*/30 phosphate buffer throughout the experiment.

As shown in Fig. 2, it is possible, then, to distinguish three groups based on the relation between R_D and R_N.

A. $R_D \fallingdotseq R_N$, peptic hydrolysis at pH 3.8 or 4.3
papaic hydrolysis at pH 5.5
tryptic hydrolysis at pH 7.4

B. $R_D < R_N$ (Dye binding ability of the splitting system decreased when compared with the rate of survival of the protein part.)
peptic treatment at pH 5.5 or 6.4
tryptic hydrolysis at pH 6.3

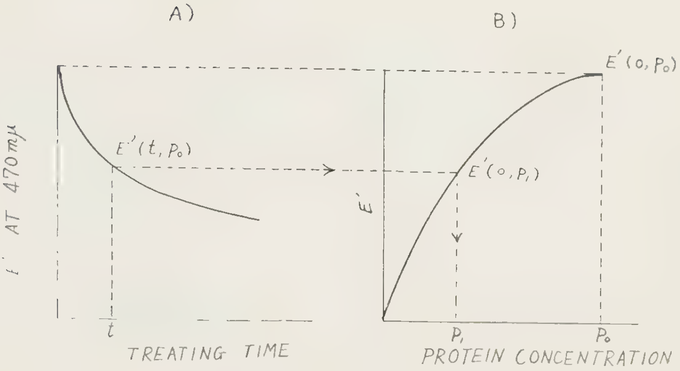

Fig. 1. Illustration of equation (1)
A) Changes in E′-value after enzymatic digestion
B) Relation between protein concentration and E′-value

C. $R_D > R_N$ (Dye binding ability of the splitting system increased more than the native protein when compared with the rate of survial of protein fraction.).
Tryptic hydrolysis at pH 8.4 or 9.0

It is concluded, from the above results, that when different enzymes attack on the same substrate, or splitting conditions are different though the combinations of substrate and enzyme are the same, different splitting products are obtained because of the difference in splitting mechanisms.

2) Characteristics of the Binding of Dyes by the Splitting Systems. As shown in Table I, molecular extinction coefficient of bound dyes and intrinsic dissociation constants of binding reaction obtained at pH 6.7 with Shimao's method were almost identical with that in the case of the binding of methyl orange by native serum albumin. Isobestic point of the absorption curve of binding system was also at 430 mμ which was the same as obtained in the case of native serum albumin.

3) Interaction between Non-protein Substances and Methyl Orange—From the spectrophotometric observation, the author could not recognize any interaction between 10^{-3} M solution of hydrochlorides of lysine, arginine or histidine and 5×10^{-5} M solution of methyl orange. Addition of the supernatants of TCA precipitation of peptic digest at pH 4.3 and tryptic digest at pH 8.4 could not give any shift on extinction coefficient of the dye.

From the experimental results of *2)* and *3)* described above, it is reasonable to consider that only the precipitatable fraction of splitting system with 0.15 N TCAA is capable of interacting with methyl orange.

4) Dye Binding Ability of the Tryptic Digesting System at pH 8.7—As mentioned above, although the methyl orange binding ability of the tryptic digesting system at pH 8.4

or 9.0 seemed to increase compared with TCAA precipitatable N content, it is necessary, in fact, to confirm whether the dye binding ability of the precipitatable fraction does increase or not. The precipitation with TCAA, however, suppress the dye binding ability of protein owing to denaturation even after redissolving of the precipitate with NaOH. The author, therefore, fractionated the hydrolyzate with saturated sodium sulfate, and the dye binding abilities of precipitatable and non-precipitatable fractions were examined. As shown in Fig. 3, the increase of dye binding ability was detectable in precipitatable fraction, E′-value was 1.5 times as large as native albumin, and the supernatant did not yeild any shift in absorption curve of methyl orange.

5) Free Arginine Content of Precipitatable Fraction with TCAA—In this journal Sakaguchi (*9*) published the method for quantitative determination of arginine, by the use of which he found that arginine content of various proteins not subjected to hydrolytic treatment was smaller than that obtained by amino acid analysis. With respect to this finding, Sakaguchi concluded that free guanidinium group of arginine in protein molecule which does not contribute to intramolecular bonding in protein is sensible to this direct method of determination without complete hydrolysis of the protein. Ac-

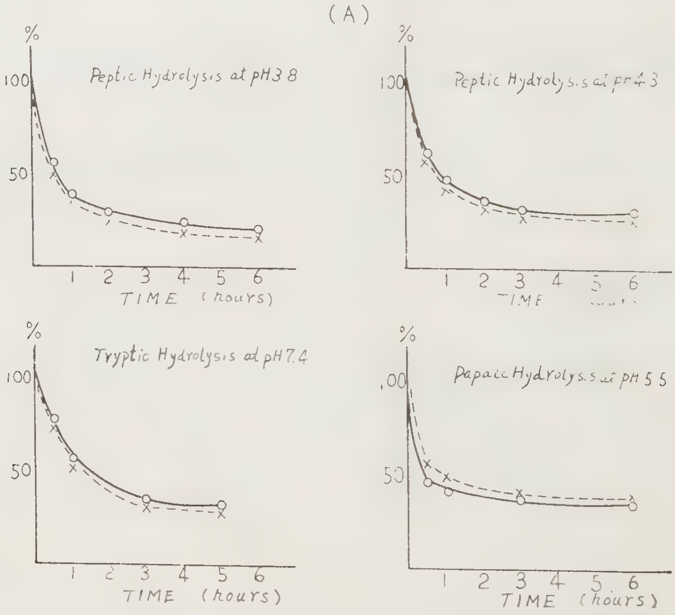

FIG. 2. Relation between R_D and R_N after enzymatic treatment of serum albumin

Continuous lines : R_D expressed as per cent.
Broken lines : R_N expressed as per cent.

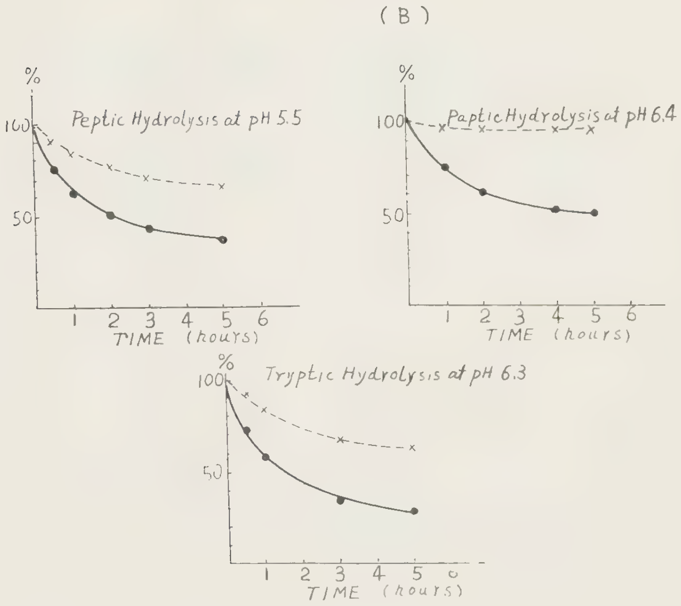

Fig. 2. B

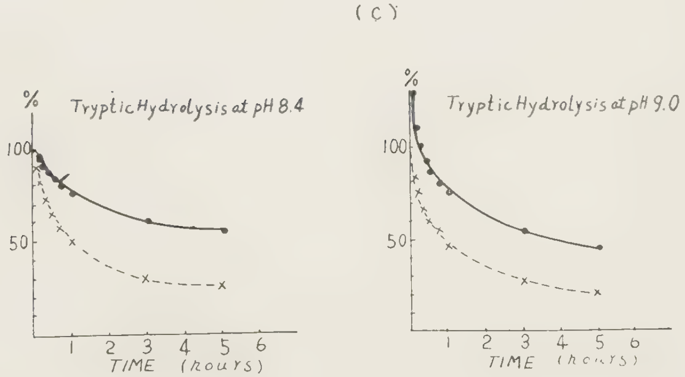

Fig. 2. C

cording to this idea, the author attempted to determine free arginine content of the protein fraction of digesting systems obtained at various conditions as the groups taking part in the binding of dyes. The protein fraction of respective digesting system was

TABLE I

Ratio of Molecular Extinction Coefficient of Free and Bound Methyl Orange and Intrinsic Dissociation Constant of Methyl Orange Binding Reaction

(pH 6.7, *M*/30 phosphate buffer)

Material	Teratment	$\varepsilon_2/\varepsilon_1$	$K \times 10^5$
Native bovine serum albumin		0.56	2.20
Tryptic hydrolyzate of albumin	pH 8.4, 3 hrs.	0.56	2.20
″	pH 8.4, 24 hrs.	0.57	2.38
″	pH 6.3, 3 hrs.	0.56	2.28
Peptic hydrolyzate of albumin	pH 4.2, 1 hrs.	0.56	2.21
″	pH 4.8, 2 hrs.	0.56	2.10
″	pH 5.8, 3 hrs.	0.56	2.35

ε_1 : Molecular extinction coefficient of free dye
ε_2 : Molecular extinction coefficient of bound dye
K : Intrinsic dissociation constant

precipitated with 0.15 *N* TCAA. Removing the supernatant, the precipitate was washed two times with 0.15 *N* TCAA by the use of centrifuge, and was redissolved with NaOH. For this solution arginine and nitrogen contents were determined with Sakaguchi's and micro-Kjeldahl methods, respectively ; and free arginine content per mg. N was computed. Free arginine (γ)/mg. N value for native albumin was calculated by the same procedure, and the ratio of the value of respective splitting system to the native albumin was shown in Table II. It is remarkable that the protein fraction of all the splitting systems contained more free arginine per N than native albumin. From this result, it is supposed that characteristics of the splitting products are different according to the difference in digesting conditions or the difference in proteolytic mechanisms.

6) *Viscosity of the Digesting Systems*—In the case of peptic or tryptic hydrolysis at pHs apart from their optimal range, the dye binding ability of the system per protein N content was obviously decreased, as shown in the results of *Expt. 1*). It is, therefore, supposed that the remained protein molecule might be subjected to a sort of denaturation so as to lose its dye binding ability. The authors attempted to determine viscosity of respective digesting system with Ostwald's viscosimeter. The ratio of the specific viscosity, η_{sp}, against the η_{sp} value at O-splitting time, η_0, were plotted in Fig. 4. In the case of peptic digestion at pH 4.3 or tryptic digestion at pH 8.4 remarkable decrease in viscosity of the splitting system accompanied by distinct hydrolysis was observed. In the case of peptic treatment at pH 6.4 or 6.9 and tryptic treatment at pH 6.3, on

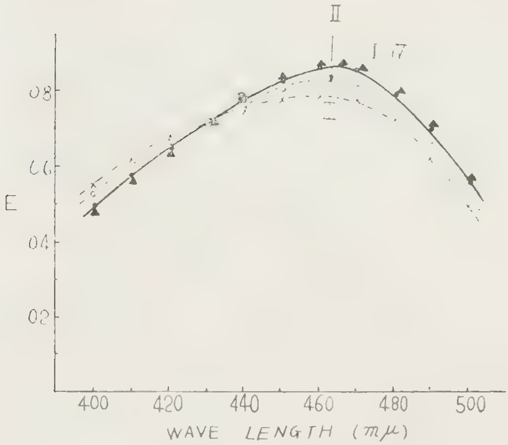

FIG. 3. Absorption specta of methyl orange

I :	●—●	in *M*/30 phosphate buffer at pH 6.7
II :	○---○	in phosphate buffer containing native albumin
III :	×-·-×	in phosphate buffer containing the precipitatable fraction with saturated Na_2SO_4 or tryptic hydrolyzate of albumin at pH 8.7 for 3 hours
IV :	▲—▲	in phosphate buffer containing the supernatant of saturated Na_2SO_4 fractionation of tryptic hydrolyzate of albumin at pH 8.7 for 3 hours

Concentration of methyl orange : $3.24 \times 10^{-5} M$ in all cases
Nitrogen content of II~IV : 3.28 mg./dl.

TABLE II

Free Arginine Content of Protein Fraction (Precipitate with 0.15 N TCAA) before and after Enzymatic Hydrolysis of Serum Albumin

Material	Treatment	Arginine (γ/mg. N)	Ratio
Before hydrolysis (control)		101	1.5
After tryptic digestion	pH 8.4, 2 hrs.	169	1.7
	pH 7.4, 2 hrs.	122	1.2
After peptic digestion	pH 5.5, 2 hrs.	123	1.2
	pH 4.3, 1 hr.	139	1.4

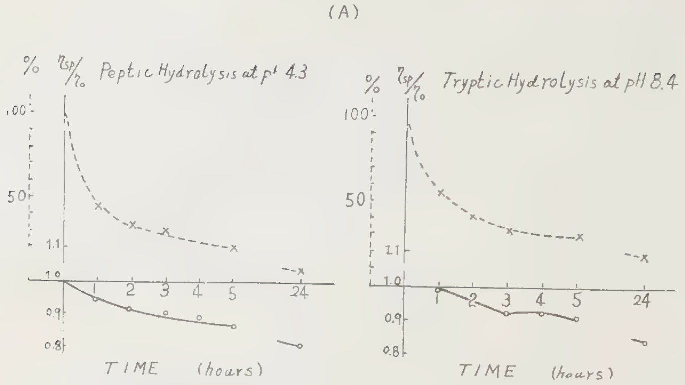

FIG. 4. Viscosity of the enzymatically treated system of serum albumin

Continuous lines : η_{sp}/η_0-value
Broken lines : R_N expressed as per cent

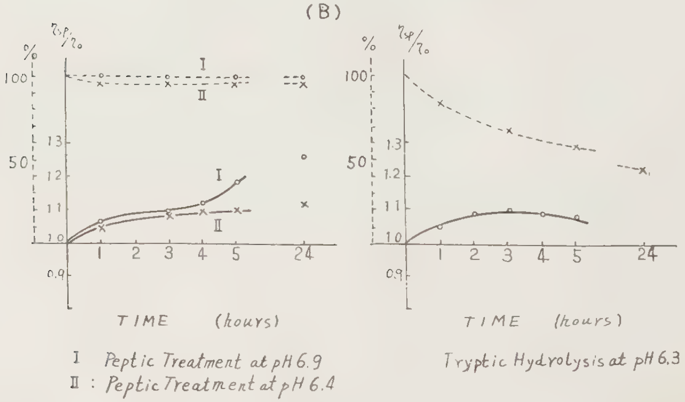

FIG. 4. B

the contrary, viscosity increased especially at the initial stage of the reaction.

DISCUSSION

By a number of investigators involving Klotz *et al.* (*2*, *3*) and the authors (*4*, *6*) free basic groups of basic amino acids, *e.g.*, lysine, arginine and histidine, on the surface of serum albumin molecule are regarded as the points taking part in the binding of the acidic dye, methyl orange. In the course of enzymatic hydrolysis of serum albumin, from basic groups described above and α-amino groups of the protein may be set free, as its globular configuration is lost and the peptide bonds are splitted; it will, therefore, be expected that the total digested system should obtain more dye binding ability than native protein. However, it was concluded, from the experimental results, that dye binding ability of the total digested system decreased in all cases, although three types were classified. Furthermore, because non protein fraction of the splitting system and simple basic amino acids did not yield any shift in extinction of methyl orange, (E′-value at 470 mμ $=0$), it is understandable that interacting with methyl orange occur only when the protein molecule is as large as precipitatable with 0.15 *N* TCAA. From the facts, on the other hand, that intrinsic dissociation constants for interactions between methyl organge and the splitting systems were the same as in the case of native albumin and the dye, it is concluded that for binding of the dye not only free animo groups of the protein but also the proper molecular weight and constant and suitable configuration arround the binding groups for the interaction are required. It is supposed that in the case of peptic digestion at pH 3.8 or 4.3 and papaic digestion at pH 5.5 only the precipitatable fraction with TCAA has the dye binding ability and the ability per N is nearly the same as that of native albumin. In the ase of the tryptic digestion at more alkaline side than pH 8.4, the optimal range for the enzyme action, the precipitable fraction with TCAA of digesting system seems to obtain the more dye binding ability than native albumin per N content. This fact suggests the increase of the groups taking part in the binding of the dye per N content, on the surface of the protein molecule unhydrolyzed by the enzyme or of the fractions holding as large molecular size as precipitatable with TCAA. And this idea fairly corresponds to the finding that free arginine content of the TCAA precipitate fraction increased more in the case of tryptic digestion at pH 8.4 than that in any other case of enzymatic treatment. However, increase of dye binding ability of the peptic digested system at pH 4.3 was not recognized although free arginine content of the precipitatable fraction with TCAA of this system increased to 1.4 times native albumin. This difference between the results in peptic and tryptic digestions must be due to the specificity of the enzymatic hydrolysis.

Referring to the experimental results of the authors described above and Bergmann's theory (*10*) introduced from their experiments on the enzymatic hydrolyses of synthetic peptides, the authors will present the following hypothesis. According to Bergmann *et al.* pepsin can attack only a peptide link lying between an L-aromatic and L-dicarboxylic amino acid, the second carboxylic radicals of which must be free (*11*, *12*) ; while trypsin can act at peptide linkages adjacent to either an arginine or a lysine unit, and the second amino groups of the diabasic amino acid unit must be unsubstituted (*13*, *14*). From this viewpoint arginine residues of the protein fraction might be hided intramolecularlly owing to steric hindrance and are not in the suitable condiguration of the binding of methyl orange even though they are set free by peptic hydrolysis. In the case of tryptic digestion, on the other hand, it might be possible that the arginine residues will be exposed at the end of molecule and there will be no steric hindrance for the binding of methyl orange. It is understandable, therefore, that free arginine might take a more suitable configuration for the binding of methyl orange after tryptic digestion than peptic hydrolysis, even though they increase to nearly the same extent in both cases. It is not yet determined whether Bergmann's ideas are capable of extending to proteins or not. But, from the author's experimental results, it seems to be reasonable to apply the Bergmann's theory for the case of tryptic hydrolysis of serum albumin in optimal range of the enzyme.

In the case of peptic or tryptic treatment of serum albumin in neutral pH range, the dye binding ability of the system per N content decreased remarkably. From this result the possibilities are conceivable as follows:

(1) Configuration of the protein fraction undigested is altered to that unsuitable for the binding of dyes ; (2) there is a fraction which has lost the dye binding ability although maintaining a large molecular size enough to be precipitatable with TCAA after partial hydrolysis ; (3) considerable aggregation of molecules in protein (precipitatable with TCAA) fraction took place. The authors, therefore, tried to determine the viscosity of the digested system and recognized its increase. If the average molecular weight becomes smaller by the enzymic hydrolysis, it is reasonably expected that the viscosity of the protein solution should decrease with time interval. After tryptic digestion at pH 8.4 or peptic hydrolysis at pH 4.3, in fact, the decrease in viscosity was noticed by the authors. However, from the fact that viscosity of the enzymatically treated system increased and dye binding of it decreased per N content

in the case of peptic or tryptic treatment of serum albumin at neutral pH range, it is supposed that aggregation of molcules in the system has occured by these enzymic actions. According to the findings of the past investigators (*15, 16*) that pepsin particularly exhibits the " Lab action ", the experimental results of the authors might be due to a kind of denaturing action of trypsin or pepsin.

The various experimental results described above suggest that determination of the dye binding ability may be capable of supplying new findings for alterations of surface configuration of protein in the course of enzymic hydrolysis, which are hardly recognized by means of other methods. If the results described in this article are referred to experimental findings by means of other methods in future, more multiple image of protein structure will be exposed before us.

SUMMARY

1. By the enzymatic hydrolysis of serum albumin, methyl orange binding ability of the digested system decreased with splitting time.

2. As follows, three groups are classified, based on the relation between R_D and R_N, where R_D is the rate of survival of the protein calculated from the decrease in dye binding ability of the digested system and R_N is that from the increase in N content of non protein fraction.

1. $R_D \fallingdotseq R_N$, peptic hydrolysis at pH 3.8 or 4.3,
   papaic hydrolysis at pH 5.5.
   tryptic hydrolysis at pH 6.4.
2. $R_D < R_N$, peptic treatment at pH 5.5 or 6.4,
   tryptic hydrolysis at pH 6.3.
3. $R_D > R_N$, tryptic hydrolysis at pH 8.4 or 9.0.

These results are considered to be due to the difference in splitting mechanisms of these enzymes.

3. Molecular extinction coefficient of bound dyes, intrinsic dissociation constants, and isobestic point of the absorption curve for the binding of methyl orange by the hydrolyzate at pH 6.7 were fairly the same as those obtained in the case of native albumin.

4. By the addition of hydrochlorides of basic amino acids of the supernatant of TCAA precipitation of the hydrolyzate to methyl orange solution, any binding reaction was not recognized. From these results, it is concluded that for binding of methyl orange not only free amino

groups of the protein but also the proper molecular weight and constant and suitable configuration around the binding sites for the interaction are required.

5. Free arginine content per N of TCAA precipitate of the hydrolyzate increased more or less than that of native albumin. From the results obtained for these arginine content and dye binding ability per N, Bergmann's idea for the specificity of the hydrolytic enzyme action upon the synthetic peptide is considered to be extendible to the case of tryptic digestion of serum albumin at optimal pH of the enzyme.

6. In the case of peptic or tryptic treatment at neutral pH range when R_D was smaller than R_N, viscosity of the hydrolyzate solution increased especially at the initial stage of the reaction. This result suggests that aggregation of remained protein molecules occurs by a kind of denaturing action of these enzymes.

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ENZYMATIC TREATMENT AND ULTRAVIOLET ABSORPTION OF SERUM ALBUMIN

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A great number of investigations (*1*, *2*) has been published about the ultraviolet absorption of protein. The absorption in wave lengths longer than 240 mμ is considered to be due to the presence of aromatic amino acids, that is, tyrosine, tryptophan, or phenylalanine in proteins. The authors (*3*) reported in the preceeding paper on the alteration in dye binding ability of serum albumin after enzymatic treatment. The present paper deals with the alteration in ultraviolet absorption spectra of the digested system caused by enzymatic hydrolysis of serum albumin, and the discussion on the mechanism of hydrolytic reactions on the basis of the results of determination of increase in the absorption of non protein fraction.

EXPERIMENTAL

Crystalline bovine serum albumin used throughout the experiments was the preparation of Armour Laboratory.

Preparations of proteolytic enzymes, pepsin and trypsin, procedures of proteolysis, and determination method of pH and nitrogen content were the same as those described in the preceeding paper.

Determination of ultraviolet absorption was carried out with Beckman's spectrophotometer (Model DU).

RESULTS

1) *Ultraviolet Absorption Spectra of Bovine Serum Albumin after Peptic Digestion*—As shown in Fig. 1, the absorption curve of native albumin solution at pH 6.7 in the range from 245 to 290 mμ was in agreement with those obtained by pervious investigators, (*1*, *2*) that is, the absorption maximum was recognized between 277 and 278 mμ, and in shorter wavelength than 245 mμ the absorption increased steeply. Alterations of this absorption curve caused by peptic digestion of the protein at pH 1.5 and 3.5 are shown in Fig. 1. That is, the absorption at shorter wavelength than 275 mμ increased from that of native albumin and at longer wavelength decreased. The absorption maximum shifted to 275 mμ and extinctions of the hydrolyzed system at various steps coincided with each other at this wavelength.

2) *Ultraviolet Absorption Spectra of Bovine Serum Albumin after Tryptic Digestion*—As illustrated in Fig. 2, increase in the absorption relative to that of native albumin was observed below 275 mμ after tryptic digestion at pH 8.7 and this tendency was the same as that in the case of peptic hydrolysis and became greater with the progress in proteolysis. In higher wavelengths the absorption did not decrease from that of native

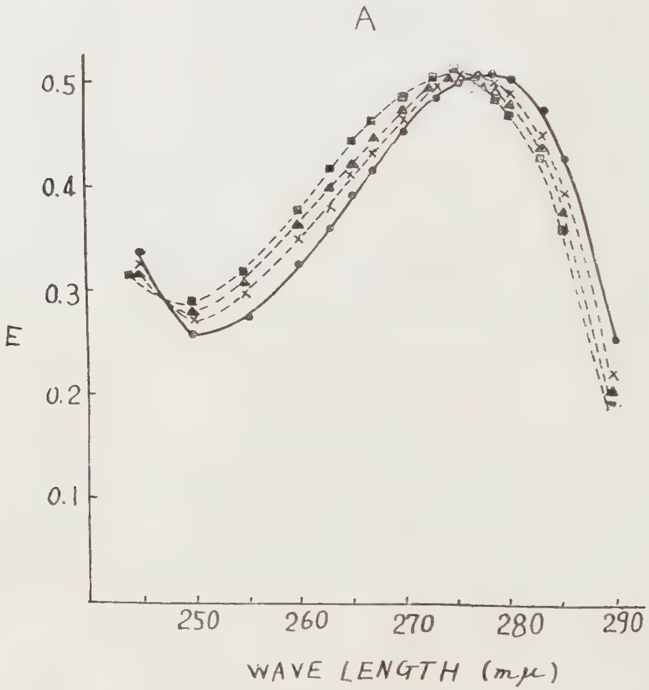

Fig. 1. A. Absorption spectrum of serum albumin before and after peptic hydrolysis (measured in $M/30$ phosphate buffer, pH 6.7)

A) peptic hydrolysis at pH 1.5

●——● before hydrolysis, protein concentration 0.07%

	S_N	initial protein concentration
× - - ×	31.5%	0.07%
▲ - - ▲	76.1%	,,
■ - - ■	98.4%	,,

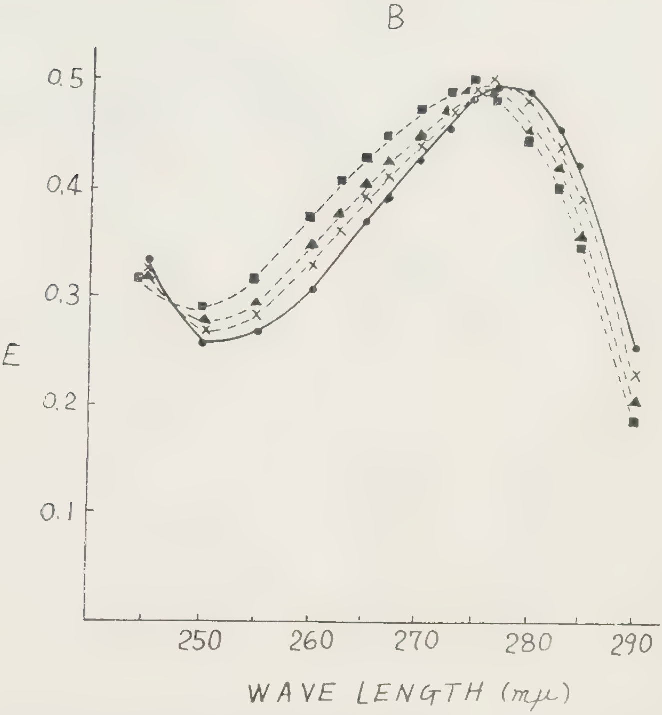

Fig. 1. B) Peptic hydrolysis at pH 3.5

●——● before hydrolysis, protein concentration 0.07%

	S_N	initial protein concentration
× - - ×	39.4%	0.07%
▲ - - ▲	71.3%	,,
■ - - ■	99.4%	,,

albumin even after sufficient treatment with trypsin, and this result was not the same as that obtained in the case of peptic digestion. Although the absorption maximum shifted to 275 mμ after considerable digestion, this shift was not recognized at initial stage of the treatment unlike that in the case of peptic hydrolysis. The absorption curves of the solutions at various steps of digestion, however, coincided with each other at 275 mμ as in the case of peptic hydrolysis.

3) Comparison between Splitting Rate Based on the Determination of Increase in Non Protein Nitrogen and That Based on the Increase in Ultraviolet Absorption of Non Protein Fraction—

a) Tryptic Digestion—In 1947 Kunitz (*4*) has published a convenient determination method of digesting rates of trypsin inhibitor by trypsin or pepsin, which based on

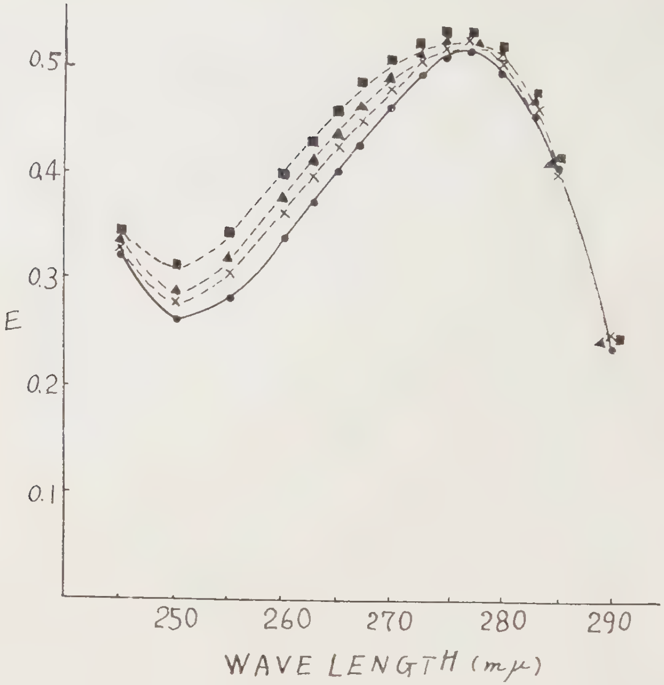

Fig. 2. Absorption spectrum of serum albumin before and after tryptic hydrolysis at pH 8.7 (measured in *M*/30 phosphate buffer, pH 6.7)

●——● before hydrolysis, protein concentration 0.07%

	S_N	initial protein concentration
× - - ×	51.8%	0.07%
▲ - - ▲	60.0%	,,
■ - - ■	70.6%	,,

increases in ultraviolet absorption at 280 mμ of non protein fraction. He considered this method as simpler and more reliable than the others. Because ultraviolet absorption curve of peptic hydrolyzate and that of tryptic hydrolyzate were different from each other, as illustrated in Expts. 1) and 2), there should be difference between the digesting rate obtained by the determination of absorption at 275 mμ of non protein fraction and that obtained from residual nitrogen determination. In order to show this difference the following experiment was performed. After adding trichloroacteic acid (TCAA), final concentration of which was 0.15 *N*, to the digested system and removing the precipitate of protein, the aliquot of the supernatant was offered to determination of nitrogen on one hand, and another aliquot from the same supernatant to determination of extinction at 275 mμ. From the results of these determinations the two rates of digestion were calculated and expressed as S_N and S_U. S_N means rate of digestion obtained by N determination and S_U that by ultraviolet absorption. As shown in Table I, S_N and S_U were in fairly good agreement in the case of tryptic digestion and, therefore, S_N/S_U-values were approximately 1.00 for all steps of hydrolysis.

TABLE I

S_N/S_U Value in the Course of Tryptic Hydrolysis of Serum Albumin

pH of treatment	Treating time	S_N*	S_U*	S_NS_U
8.7	*min.* 5	31.1	30.5	1.01
	10	40.1	39.9	1.00
	15	46.6	45.7	1.02
	30	51.8	50.8	1.03
	60	60.0	60.0	1.00
	180	70.6	70.0	1.01
7.4	30	25.2	25.7	0.98
	60	42.3	41.9	1.01
	120	60.5	61.1	0.99
	180	71.2	71.4	1.00
	300	72.5	72.7	1.00

* Percentage hydrolysis

TABLE II

S_N/S_U Value in the Course of Peptic Hydrolysis of Serum Albumin

	S_N*			S_U*			S_N/S_U		
Time \ pH	1.5	2.7	3.8	1.5	2.7	3.8	15.1	2.7	3.8
min. 5	52.4	48.1	26.3	43.2	34.4	11.5	1.21	1.40	2.30
10	75.2	65.2	42.5	66.5	49.4	29.9	1.13	1.30	1.41
15	82.6	72.8	59.0	75.9	61.0	44.4	1.09	1.19	1.33
30	92.4	89.2	70.0	92.6	79.0	60.3	0.99	1.13	1.16
60	99.4	98.4	85.0	99.6	91.7	76.7	0.99	1.07	1.10
180		98.0	95.5		98.3	100.0		0.99	0.96

* percentage hydrolysis

b) *Peptic Digestion*—In the course of peptic hydrolysis S_N- and S_U-values obtained by the same procedure as that in the case of tryptic digestion were not in agreement, as shown in Table II. Differences between the two values were recognized only at initial stage of the treatment at pH 1.5, and from the beginning to considerably later stage of hydrolysis at pH 2.7, and 3.8. That is, at initial stage S_N was greater than S_U and, therefore, S_N/S_U values were greater than 1.00. The S_N/S_U-value, however, approached to 1.00 gradually and after the complete hydrolysis of the protein, as indicated by the formation of no precipitate by addition of TCAA to the digest, S_N- and S_U-values became to be in complete agreement with each other. And in the case of this peptic digestion, unlike in tryptic hydrolysis, absorption maxima of the non protein fractions of hydrolyzate were situated at 275 mμ from the initial stage of hydrolysis.

Thus, the results obtained by determination of S_N- and S_U-values also indicated the presence of difference in mechanisms of proteolytic actions of pepsin and trypsin.

DISCUSSION

The experiments on the changes in ultraviolet absorption of serum proteins after enzymatic hydrolysis have been performed by several investigators. It was mentioned by Haurowitz (*5*) that the absorption curve for genuine and hydrolyzed protein was about equal. But the absorption curve for the peptic digested serum globulin obtained by Holiday *et al.* (*1*) was of approximately the same nature as that for the peptic digested serum albumin obtained by us. Comparing the absorption of bovine serum albumin with that of a mixture of tyrosine, tryptophan, and phenylanine corresponding to the quantities of these amino acids in the protein, Rideal, Roberts (*6*), and Sizer and Peacock (*7*) indicated that in the 250∼275 mμ region both absorptions were nearly equal and at higher wavelengths the absorption of amino acid mixture was weaker than that of the protein. In addition it was described by them that absorption maximum was situated at 277∼278 mμ in the protein, and at 275 mμ in the amino acid mixture. Although the experimental results obtained by us showed that the absorption of serum albumin at lower wavelengths than 275 mμ increased remarkably after considerable proteolysis with both pepsin and trypsin, the absorption of the hydrolyzate in this range might became entirely equal to that of native protein, as stated by Rideal *et al.*, if these enzyme completely converted their substrate to constituent amino acids alone. Although it is supposed by Schauenstein *et al.* (*8, 9*) that the absorption of protein in this range may be due to interchain hydrogen bond, there is not any decisive information for supporting this imagination. As this supposition is considered to be hardly capable of application especially for globular protein such as serum albumin, a meaning of the increases in absorption at this range is yet obscure. The fact that maximum absorption shifted to 275 mμ and absorption in longer wavelengths than this point decreased from that of native albumin in the course of peptic digestion is in agreement with the results obtained for amino acid mixture by Rideal *et al.*. On the contrary, the absorption curve of the tryptic digest is identical with that of native albumin so far as in the higher wavelengths than 275 mμ. Referring to the Bergmann's theory (*10*) for specificity of hydrolytic enzyme action upon synthetic peptide that pepsin attacks on a peptide bond lying between an aromatic and an acidic amino acids, it is likely that after peptic digestion resulting absorption curves in the ultraviolet resemble those for amino acid mixture more closely than those obtained after tryptic digestion. The fact that S_N- and S_U-values are in fairly agreement from initial period of tryptic digestion and are not equal in the case of peptic hydrolysis is also explained by Bergmann's theory as follows. Because trypsin acts on peptide bonds unrelated to aromatic amino acids, aromatic amino acids are sent into non protein fraction accompanied by nitrogen in the ame rate in the course of the enzymatic hydrolysis and consequently S_N- and S_U-values are equal. On the contrary, in the case of peptic hydrolysis, because the enzyme attacks only the peptide bond adjacent to aromatic amino acid, the hydrolysis of serum albumin starts in the mode that aromatic amino acids are left in the TCAA precipitatable fraction per N content and so S_N- values became larger than S_U.

From the results obtained in the present and the recent paper on the dye binding ability and the ultraviolet absorption of peptic and tryptic digestion of serum albumin, the Bergmann's theory seems to be applicable for the interpretation of the reaction mechanism of both tryptic and peptic hydrolysis of serum albumin.

SUMMARY

1. The ultraviolet absorption curves were obtained by the use of Beckman's spectrophotometer for native serum albumin and peptic or tryptic digest of the protein. In the case of peptic digestion, the absorption at shorter wavelengths than 275 mμ increased from that of native albumin and at longer wavelengths decreased. After tryptic digestion the absorption at shorter wavelengths than 275 mμ increased but at longer wavelength identical with that of native albumin. After

hydrolysis by pepsin or trypsin absorption maximum shifted to 275 mμ and extinctions of the digested systems of various steps were in coincidence at this wavelength.

2. In the case of tryptic digestion, S_N- and S_U-values were in agreement, where S_N and S_U were the splitting rates of the protein based on increases in non protein nitrogen and in ultraviolet absorption of non protein fraction, respectively.

3. In the case of peptic digestion, S_N was larger than S_U especially at the initial stage of hydrolysis. This difference in the two splitting rates might be due to the difference in the mode of actions of pepsin and trypsin. And Bergmann's theory for the specificity of these enzymes to synthetic peptide was applied for the interpretation of this phenomenon.

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AMMONIA FORMATION AND GLUTAMIC ACID IN BRAIN TISSUE

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The formation of ammonia in brain tissue is physiologically important in relation to the functional metabolism. It is known that the content of ammonia in brain greatly increases during seizures induced by electrical stimulation or by many kinds of convulsants (picrotoxin, pentamethylentetrazol or fluoroacetate) (*1*). Richter and Dawson (*2*) suggested that convulsions might be attributed directly to the irritating action of ammonia, because the injection of large doses of ammonium salts could induce convulsions and also because the liberation of ammonia occurred already in the preconvulsive state after picrotoxin administration or electrical stimulation.

It is known that, in frog nerves, the liberation of ammonia is also increased by the electrical stimulation and it has a close relation to the physiological function of nerve (*3*). This also occurred in squid fibers and it was suggested that the underlying mechanism of ammonia formation in nerve fibres would be similar to that in brain tissue (*4*).

The mechanism and sources of ammonia formation in brain tissue have been quite obscure, though many possibilities have been considered (*5*). The ammonia-producing enzymes known to occur in brain tissue are mainly (1) glutamic dehydrogenase, (2) adenylic deaminase and (3) glutaminase. These three enzymes are believed to be the most likely physiological agents of rapid formation of ammonia, and other factors, for example, amine oxidase, can be eliminated with a fair degree of probability as important sources. Richter and Dawson (*2*) found that the normal ammonia content in brain was very low, but that it rapidly increased to about 1 mg. per cent in 30 seconds after decapitation. Thus, the formation of ammonia after decapitation is almost explosive in brain tissue.

These considerations prompted the analyses of the above three enzymatic reactions in excised brain tissue to find out the immediate precursors and mechanism of ammonia formation. The increase of ammonia was accompanied by the great decrease of glutamic acid content, and in organized brain preparations the adenylic deaminase was entirely inactive. Accordingly, it was thought that the main source of rapid ammonia formation in brain was glutamic acid.

A preliminary report on a part of this investigation has been published elsewhere (*6*).

METHODS AND MATERIALS

Guinea pigs weighing from 150 to 200 g. were used exclusively.

Brain Homogenate—The head of guillotined animal was instantaneously immersed in liquid air to freeze it solid in a few seconds and not to produce any changes of metabolites in brain tissue. The completely frozen brain was rapidly removed and crushed to a powder, about 400 mg. of which was transferred to a previously weighed cup containing 2 ml. of modified Krebs-Ringer phosphate solution. It was immediately weighed as a whole, and the tissue weight was given from the difference. And then, it was incubated in absence of specific substrates at 37°. The composition of original Krebs-Ringer solution (*7*) was modified and the concentration of sodium chloride and potassium chloride was exchanged to assimilate the medium to the intracellular condition, and pH was adjusted to 7.0.

After the required incubation time, the same volume of 20 per cent TCA* was added. The mixture was centrifuged (not filtered) and the estimations were carried out on the clear supernatant. Of course, when the starting values were to be determined, the crushed brain was directly transferred to 10 per cent TCA.

Brain Slice—The animal was bled from the carotids, and the brain was excised. The slice was cut free-hand with a safety razor on a cold stage. Wet weight was known by a torsion-balance. The incubation medium was original Krebs-Ringer phosphate solution (*7*) of pH 7.0. After the incubation, the reaction was stopped by the addition of the same volume of 20 per cent TCA at 0°, and it was homogenized with a Potter homogenizer (*7*). The homogenized slice was centrifuged and the supernatant was taken.

Estimations—The ammonia content in TCA extract was estimated by Conway's method of microdiffusion analysis (*8*). Glutamine was determined by the method of Richter and Dawson (*2*), which depends on estimating the ammonia liberated on hydrolysis under standard conditions with 10 per cent TCA at 70° for 75 minutes. Glutamic acid was estimated manometrically, using a decarboxylase preparation of Japanese squash. Similarly to the observation by Okunuki (*9*), the enzyme prepared from the squash cortex did not decarboxylate glutamine, aspartic acid and α-ketoglutaric acid and was quite stable. His procedure was strictly followed for its preparation. The extraction of glutamic acid from the brain tissue samples for its estimation was carried out by the method of Krebs (*10*).

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* The following abbreviations are used throughout : TCA, trichloroacetic acid ; ATP, adenosinetriphosphate ; AMP, muscle adenylic acid ; ITP, inosinetriphosphate ; IMP, inosinc acid.

Adenylic compounds were determined in a Beckman model DU quartz spectrophotometer in a 5 per cent perchloric acid extract.

From the extinction at 260 mμ, the molecular concentration of adenylic compounds was given (*11*). When an adenine ring was deaminated producing a hypoxanthine, the absorption curve clearly changed and the degree of deamination was given from the difference of extinction at 265 mμ (*11*).

Chemicals—ATP was isolated from rabbit muscle by a modification of the method described by Le Page (*12*). It was checked chromatographically using an ion-exchange resin (Dowex-1, 200–400 mesh) by the method of Cohn and Carter (*13*), and its purity was about 78 per cent. AMP and ITP were prepared from ATP by the usual methods (*7*). IMP was extracted directly from rabbit muscle by the method of Marmur *et al.* (*14*). Identification of these nucleotides was carried out from their ultraviolet absorption curves and phosphorus contents. Other reagents used were commercial products.

RESULTS

Homogenate—The relationships between the changes of contents of ammonia, glutamine, glutamic acid and adenylic compounds during the incubation without specific substrates are summarized in Fig. 1.

After 30 minute incubation, ammonia was formed considerably, and at the same time the glutamic acid content decreased, but glutamine rather increased. Adenylic compounds were deaminated as indicated in Fig. 2. These results show that the glutamic acid—glutamine system under the conditions investigated was functioning as an ammonia-binding mechanism rather than as an ammonia-forming system and both glutamic acid and adenylic compounds are considered to be the ammonia sources.

However, after 10 minute incubation, the considerable ammonia formation was accompanied by the significant decrease of glutamic acid content without the deamination of adenylic compounds. Therefore, it is shown that the main source of ammonia during the early stage was glutamic acid, and the deamination of adenylic compounds was a slow process.

When L-glutamic acid was added, the formation of ammonia was rather increased showing that both the glutamic acid deamination and glutamine synthesis were increased by added glutamic acid. But when L-glutamic acid was added to the brain homogenate which was prepared in an ice-cold bath, the formation of ammonia decreased and there was a significant increase of glutamine (Table I). It is probably due to the deficiency of diphosphopyridine nucleotide which is known to be rapidly broken down enzymatically in brain homogenate (*15*).

Ammonia content before incubation was very low as already shown by Richter

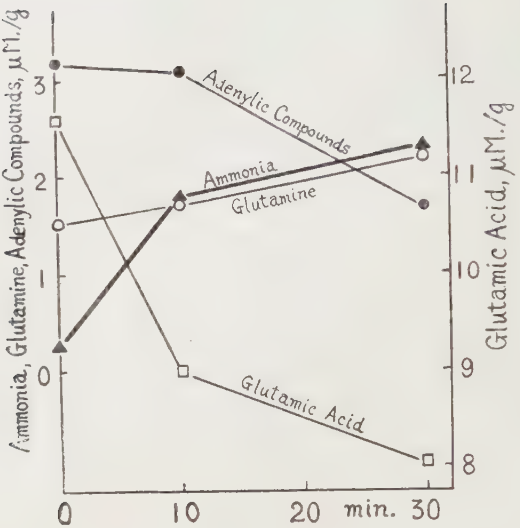

FIG. 1. Ammonia formation and related substances in brain homogenate.

TABLE I

Ammonia Formation in Brain Homogenate in the Presence of M/50 Glutamate

	Liquid air		Ice-cold bath	
	Control	Glutamate	Control	Glutamate
NH_3-nitrogen	3.14	3.71	5.62	3.65
Glutamine	32.7	41.3	62.5	110

Amounts of NH_3-nitrogen and glutamine after 30 minute incubation at 37° are shown in mg. per cent.

and Dawson (*2*), but the present values were slightly higher than theirs because the head of experimental animal was frozen in liquid air after decapitation. The amount of glutamine was significantly lower than their values, of which reason is quite obsqure, but in the homogenate prepared in an ice-cold bath it resembled theirs.

Slices—Table II shows the ammonia formation and the changes of related com-

TABLE II

Ammonia Formation in Brain Slices

Incubation time *min.*	0	40
NH$_3$-nitrogen	1.45	2.29
Glutamine	39.0	41.5
Glutamic acid	112	91
Adenylic compounds	2.1	2.0

See the text for the experimental condition. NH$_3$-nitrogen and glutamine are shown in mg. per cent, and glutamic acid in mg. per cent as sodium salt. The amount of adenylic compounds is shown in μM per g.

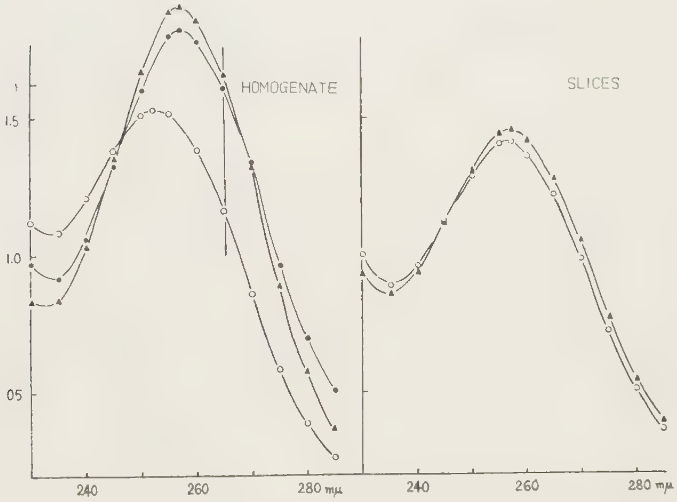

FIG. 2. Ultraviolet absorption curves of brain tissue.
▲, 0 min. ; ●, 10 min. at 37° (omogenate only) ; ○, 50 min. at 37°

pounds in brain slices. In them, ammonia formation was also accompanied by the glutamic acid decrease, but the glutamine content was rather increased. In slices, unlike in homogenate, the adenylic compounds were not deaminated during the incubation of 50 minutes (Fig. 2). It is interesting that the adenylic deaminase was apparently inactive in spite of its clear occuring in homogenate.

Metabolism of Adenylic Compounds—When AMP was added to brain homogenate, the ammonia formation increased markedly, but when added to brain slices, there was rather a decrease in ammonia formation. These results are in good agreement with the findings obtained under substrate-free conditions. Accordingly it is shown that the adenylic deaminase is inactive in organized brain preparations.

Recently, Weil-Malherbe (*5*) suggested the amination of inosinic acid by glutamine at the expense of high energy phosphate bonds and the ammonia formation from glutamine through adenylic acid. The increase of ammonia formation due to ITP accompanied by the decrease of glutamine content was confirmed in brain slices. However, because the adenylic deaminase was inactive in brain slices and not activated by ITP, the increase of ammonia formation in this case is considered simply due to the activation of glutaminase. IMP had not the same effect as ITP (Table III).

TABLE III

The Influence of Nucleotides on Ammonia Formation in Brain Slices

Condition	NH_3-nitrogen	Glutamine
	mg. %	*mg. %*
Control: 40 min., 37°.	4.23	37.9
AMP, 10^{-3} *M* added.	4.16	40.2
ITP, 10^{-3} *M* "	5.76	19.0
IMP, 10^{-3} *M* "	3.42	33.0
AMP, 10^{-3} *M*+ITP, 10^{-3} *M* added.	5.28	17.4

Moreover, the amination of inosinic acid was not detected spectrophotometrically, when ITP and glutamine were incubated with brain slices. Therefore, adenylic acid —inosinic acid transformation is not believed to be the active process in brain slices.

DISCUSSION

The special physiological significance of glutamic acid in brain metabolism is suggested by many investigations in recent years which were carried out *in vitro* as well as *in vivo* (*16*). Ammonia is a powerful cerebral irritant, and Richter and Dawson (*2*) thought that ammonia might play a role in the precipitation of epileptic seizures, though the increase of ammonia in the cerebrospinal fluid of epileptics after seizures was disproved by Richter *et al.* (*17*). It is generally believed that the enzyme systems centered around glutamic acid are functioning for the neutralization and disposal of ammonia (16). The report that glutamic acid reduced the incidence of attacks of petit mal in epileptics

(*18*) may be significant in this connection. On the other hand it was also reported that the epileptiform convulsions were induced by glutamic acid which was injected directly into cerebral cortex of dogs (*19*). This apparent discrepancy on the action of glutamic acid was fully discussed by Hayashi (*20*). It is very interesting, therefore, that glutamic acid is concerned directly with ammonia formation systems in excised brain tissue.

The ammonia formation in brain tissue has generally been considered to come from the deamination of nucleotides, though it has been regarded as a relatively slow process, because glutamine was found to produce no ammonia by many works. In fact, adenylic compounds are deaminated in brain homogenate as shown in the present work. Also, Muntz (*21*) reported that brain tissue contained a specific adenylic deaminase other than Schmidt's deaminase. But the fact that in organized brain tissue adenylic deaminase is quite inactive is very interesting, because it disproves the general hypothesis that ammonia in brain comes from the adenylic compounds. Moreover, it shows that the enzyme activity is influenced by the cell structure, and an active enzyme system in homogenate or extract is not necessarily active in an organized cell structure.

Weil-Malherbe (*22*) pointed out that the large ammonia formation of brain slices which reached to as much as 56 mg. per cent in 4 hours could not be accounted for by low molecular cell constituents. Actually, he showed that the proteolytic reactions contributed partly to ammonia formation (*5*). But, under the conditions of the present investigation, especially in the early stage, the autolysis of high molecular substances would be neglected as a possible source of ammonia.

So far as the decrease of glutamic acid content under the present conditions is concerned, the reactions of transaminase and glutamic decarboxylase besides the α-ketoglutaric acid—glutamic acid—glutamine system should be taken into consideration. But such a great decrease of glutamic acid would not be explained without considering the oxidative glutamic deamination. The analysis of transaminase system in brain tissue is now in progress.

From the above results it is quite probable that the ammonia formation in organized brain tissue comes mainly from glutamic acid deamination. It suggests that, in conditions such as electric shock, in which the ammonia content of brain is rapidly increased, the increased ammonia formation may be due to glutamic dehydrogenation. In fluoroacetate poisoning, convulsions are accompanied by the increase of ammonia (*23*) and at the same time by the decrease of glutamic acid (*24*). It is possible to consider that the glutamic acid disappearing is oxidized and donates α-ketoglutaric acid to the tricarboxylic acid cycle with the consequent production of more energy in such emergencies.

SUMMARY

1. In brain homogenate, the ammonia formation without specific substrates is accompanied by the great decrease of glutamic acid content. In the first 10 minutes, glutamic acid is decreased considerably, but adenylic compounds are scarcely deaminated and glutamine is rather increased. These facts show that the main source of ammonia formation during the first 10 minutes is glutamic acid.

2. Brain slices does not deaminate adenylic compounds contained, and moreover, added adenylic acid is not changed over into inosinic acid. It is concluded that adenylic deaminase is inactive in slices. The transformation between adenylic acid and inosinic acid is not considered to be an active process in brain.

3. The problem of ammonia formation in brain was discussed from its great physiological significance, with special reference to the glutamic acid metabolism.

The author expresses his appreciation to Prof. G. Kato and Prof. T. Hayashi for their interest and encouragement during the course of this work and to Dr. Y. Tsukada for his many suggestions and criticism.

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STUDIES ON CHOLESTEROL ESTERASE OF THE ORGANS PRODUCING STEROID HORMONES IN THE RAT

I. ITS PRESENCE IN THE SUPRARENAL BODY

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The presence of cholesterol esterase has been established in liver (*1*), spleen (*1*), kidney (*2*), pancreas (*2*), intestinal mucosa (*2*), intestinal juice (*3*), bile (*4*), serum (*5*) and in adipose tissue (*6*). It is assumed that this enzyme does not only hydrolyze esterified cholesterol but esterify free cholesterol. And both hydrolytic and esterifying cholesterol esterase systems have been postulated to play not a few important role on the mobilization of cholesterol and of fatty acids in the animal body.

It is well known fact that the organs producing steroid hormones such as suprarenal body, testes or ovary are abundant in esterified cholesterol. Recently many works have been made on the possibility of the conversion of cholesterol in these oragns to steroid hormones and this possibility has been indirectly proved by many workers. The author (*7*) reported the changes of cholesterol content in the reproductive organs of the rats which had shown the dysfunction of reproduction caused by the deficiency of polyunsaturated fatty acids in the diets. It may throw some light on the mechanism of the action of polyunsaturated fatty acid to try the detailed investigation of these changes, especially mobilization of cholesterol. From this point the author has undertaken an investigation of the presence of cholesterol esterase in the reproductive organs.

Simola (*8*) observed that there existed a certain enzyme which has the synthetic action of esterified cholesterol in the suprarenal bodies of cattle, horse, sheep, and swine, but we have as yet very little information as to what is involved in the presence of such enzyme in the suprarenal body of the rat, and further in the presence of cholesterol esterase in the testes or the ovary of the rat. In the studies reported in this paper the work of Simola was extended and the esterifying and hydrolytic cholesterol esterase systems in the suprarenal body of the rat were established.

METHODS AND RESULTS

The test animals used were both male and female Wistar strain adult rats in body weight 180∼240 g. fed with unhulled wheats, green vegetables, salts and water in the author's laboratory. These animals were killed by decapitation under ether anaesthesia. The suprarenal bodies of the animals were removed immeadiately after death and homogenized in Krebs buffer in a homogenizer. Five ml. of the homogenate, equivalent to 5 mg. of suprarenal body, was used as an enzyme source.

Inactive human serum by warming at 58° for 1 hour was prepared as the substrate of the above enzyme preparations. Cholesterol content of the substrate is 162∼245 mg./dl., 60.4 per cent of which was esterified form.

To the mixtures of 3 ml. of the serum, 0.9 ml. of Sörensen buffer and sodium glycocholate (25∼100 mg.), 3.0 ml. of the homogenate was added. The ratio of free cholesterol was immediately determined in 2.3 ml. of the homogenate mixtures, which corresponds with 1 ml. of the serum. The rest of the homogenate mixtures was incubated on a shaking drum in a water bath at 38.0°. To test esterifying function of the homogenate mixtures, samples were removed from the homogenate mixtures at 3, 6, 12 and 24 hours after incubation.

Total and free cholesterol fractions were determined by the method of Schoenheimer and Sperry (*9*).

Influence of pH—Sörensen buffer solution of various pH, the values of which were adjusted by Beckman pH meter modeled G, was prepared to search the optimum pH of adrenal cholesterol esterase. The result is shown in Fig. 1. The figure represents that the optimum pH of cholesterol esterifying activity of the adrenal homogenate stands between 6.8 and 7.0 (6.9). And any difference of the value was not seen between male and female adrenals. The degree of esterifying activity increased after 24 hours of incubation at pH 6.9 and its value reached at about 30 per cent. When the homogenate mixtures were boiled at 60° for 20 minutes, their esterifying activity was completely destroyed. In these tests it is found that the nature of cholesterol esterifying activity of the rat adrenal homogenate seems to be enzymatic.

Influence of Activator—Nedswedski (*10*, *11*) showed that the bile salts exert their effect by stabilizing pancreatic cholesterol esterase. Klein (*12*) also observed that hydrolytic cholesterol esterase of cattle pancreas was activated by cholic acid. The activating effect of bile salts to hydrolytic and esterifying cholesterol esterase systems was demonstrated in pancreas (*13*), dog serum (14), rat intestinal mucosa (*15*) and rat adipose tissue (*16*). Experiment were done to analyze the effect of bile salts to the adrenal homogenate of the rat. Esterifying activity of the rat adrenal homogenate to the inactive human serum under various concentrations of sodium glycocholate is shown Table I. Table II shows the result obtained from the experiment on the hydrolytic enzyme system. In both cases sodium glycocholate itself has no action in any con-

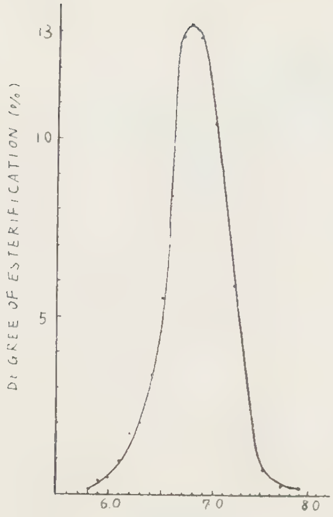

Fig. 1. Esterifying activity of adrenal cholesterol esterase under various pH (Substrate : Inactive human serum, 38°, 4 houors incubation)

centrations.

It is quite clear that cholesterol esterifying function of the adrenal homogenate increases in proportion to the concentration of sodium glycocholate. And also in this experiment there is no difference between male and female rats.

Esterifying Function—The results determined the cholesterol-esterifying effect of the rat adrenal homogenate using the inactive human serum by the method of Metzger (*16*) and using the artificial substrate (cholesterol and sodium oleate) by the method of Treadwell *et al.* (*15, 18*) are shown in Tables III and IV respectively. These tables show clearly that adrenal cholesterol esterase of the rat esterifies the inactive human serum as well as artificial substrate. And it is to be noted that there is not any differences between male and femal rarts in esterifying function of adrenal cholesterol esterase.

Hydrolytic Function—Cholesterol esterase in various tissues exerts both hydrolytic and esterifying effects. This experiment was made to investigate the hydrolytic function of rat adrenal cholesterol esterase. The hydrolytic degree of rat adrenal cholesterol esterase used inactive human serum as substrate in the course of time of incubation is demonstrated in Table V. And Table VI shows hydrolysis of an artificial substrate. The artificial

Table I

Activation of Esterfying Adrenal Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.9, 38°, 4 hrs. incubation)

Concn. of Na glycocholate in serum	Rat No. Sex	Degree of esterification 301	302	303	304	5298	5299	5300	5301	Average
		Male				Female				
‰		%	%	%	%	%	%	%	%	%
0		4.7	2.1	7.2	10.2	3.0	10.9	4.5	2.6	5.65
10		5.3	2.5	9.4	10.9	3.7	12.8	6.1	3.1	6.73
20		7.8	4.7	11.2	13.4	5.6	14.2	8.4	5.8	8.89
30		11.8	9.7	15.2	16.4	10.2	17.1	14.3	8.2	12.86

TABLE II

Activation of Hydrolytic Adrenal Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.9, 38°, 4 hrs. incubation)

Concn. of Na glycocholate in serum	Rat No.	Degree of hydrolysis								
		305	308	309	310	5302	5303	5305	5306	Average
	Sex		Male				Female			
‰		%	%	%	%	%	%	%	%	%
0		0.2	1.5	2.7	2.1	1.8	2.1	0.9	1.4	1.59
10		0.8	1.9	3.5	2.6	2.5	2.7	1.1	2.0	2.14
20		1.5	2.4	4.7	3.4	2.8	3.5	1.4	2.9	2.83
30		2.3	3.0	5.1	4.6	4.2	4.5	2.0	3.8	3.89

TABLE III

Esterification of Inactive Human Serum by Adrenal Cholesterol Esterase

(pH 6.9, 98°, Na glycocholate 30 mg.)

Rat No.	Sex	Degree of esterification			
		3 hrs.	6 hrs.	12 hrs.	24 hrs.
		%	%	%	%
306		8.3	9.1	9.9	10.7
307	Male	15.2	16.3	18.7	21.6
311		14.9	15.5	18.9	27.2
5308		17.1	18.2	22.7	30.3
5310	Female	9.0	9.2	10.6	11.4
5311		11.4	13.5	17.8	22.5

TABLE IV

Esterification of Cholesterol with Sodium Oleate by Adrenal Cholesterol Esterase

(pH 6.9, 38°, Na glycocholate 30 mg.)

Rat No.	Sex	Degree of esterification			
		3 hurs.	6 hrs.	12 hrs.	24 hrs.
		%	%	%	%
315		6.7	7.9	9.0	11.3
316	Male	14.3	15.9	18.1	24.3
318		10.8	12.1	15.8	19.8
5314		5.9	7.0	8.8	11.8
5315	Female	11.1	15.9	19.3	23.7
5316		13.2	16.2	20.1	26.6

TABLE V

Hydrolysis of Inactive Human Serum by Adrenal Cholesterol Esterase

(pH 6,9, 38°, Na glycocholate 30 mg.)

Rat No.	Sex	Degree of hydrolysis			
		3 hrs.	6 hrs.	12 hrs.	24 hrs.
		%	%	%	%
329	Male	3.8	4.1	4.7	5.7
330		4.7	5.2	5.7	8.0
332		5.1	5.8	6.0	6.3
5330	Female	5.2	6.3	6.9	10.7
5331		3.2	3.4	3.5	3.8
5332		4.4	4.7	5.2	7.1

TABLE VI

Hydrolysis of Cholesteryl Oleate Emulsion by Adrenal Cholesterol Esterase

(pH 6,9, 38°, Na glycocholate 30 mg.)

Rat No.	Sex	Degree of hydrolysis			
		3 hrs.	6 hrs.	12 hrs.	24 hrs.
		%	%	%	%
325	Male	3.0	3.8	4.1	4.9
335		4.3	4.9	5.4	7.1
336		1.2	1.5	2.2	2.8
5333	Female	2.1	2.1	3.0	3.6
5334		3.8	4.5	5.1	7.0
5335		1.6	2.2	2.6	2.7

substrate was prepared from sodium oleate by the method of Schoelly (17). It is readily seen that adrenal cholesterol esterase of the rat in both sexes has not only esterifying activity but also hydrolytic activity, and that the latter is remarkably inferior to the former.

Influence of the Concentration of the Enzyme—It is assumed that enzymes are considered organic catalysts and what is known of the laws of catalysists can be applied to enzymes. The enzymes are often inhibited in their activity by increasing the quantity of substrate, and are also inhibited in their activity by the accumulation of the products of the reaction. The following experiments were carried out for searching whether adrenal cholesterol esterase is fellen under the same rules or not.

At first the proportionality between the concentration of the enzyme and the degree of the reaction was measured by esterification of 3.0 ml. of the inactive human serum at pH 6.9 for 4 hours incubation with the addition of 25 mg. sodium glycocholate. This result is shown in Table VII. The equal result was obtained in the hydrolytic adrenal cholesterol esterase system (Table VIII).

TABLE VII

Influence of the Quantity of Adrenal Cholesterol Esterase on Its Esterifying Activity

(pH 6.9, 38°)

Male Rat No.	Female Rat. No.	Quantity of adrenal homogenate		Esterification after 4 hrs. incubation (av. values)
338, 339	5339, 5340	0 *ml.*		0 %
338, 339	5339, 5340	1		3.15
339, 343	5340, 5342	2		12.68
343, 344	5342, 5347	5		36.47
344, 347	5347, 5348	10		78.53
342, 347	5342, 5348	5	boiled at 60° for	0
338, 339	5339, 5340	10	20 minutes	0

TABLE VIII

Influence of the Quantity of Adrenal Cholesterol Esterase on Its Hydrolytic Activity

(pH 6.9, 38°, Substrate : Inactive human serum, Na glycocholate 25 mg.)

Male Rat No.	Female Rat. No.	Quantity of adrenal homogenate		Hydrolysis after 4 hrs. incubation (av. values)
350, 351	5349, 5352	0 *ml.*		0 %
350, 351	5349, 5352	1		1.15
351, 352	5352, 5353	2		4.33
351, 355	5353, 5354	5		5.82
355, 356	5354, 5355	10		7.06
350, 356	5355, 5356	5	boiled at 60° for	0
355, 359	5352, 5356	10	20 minites	0

Next the inhibition by the procudts of the reaction (cholesteryl oleate) was observed by esterification of the inactive human serum plus 25 mg. of sodium glycocholate under various concentrations of sodium oleate. The result is shown in Table IX. And the inhibition of hydrolytic adrenal cholesterol esterase system by sodium oleate was seen in Table X.

TABLE IX

Influence of Cholesteryl Oleate on the Esterifying Activity of Adrenal Cholesterol Esterase

(pH 6.9, 38°)

Concn. of cholesteryl oleate	Time of incubation	Degree of esterification (average values)		
		Expt. I (5 males & 5 females)	Expt. II (5 males & 5 females)	Expt. III (5 males & 5 females)
0.0 %	1 *hrs.*	6.77%	4.19%	7.03%
	4	12.38	10.45	14.62
0.01	1	3.21	3.17	4.89
	4	8.32	7.12	10.75
0.05	1	2.23	1.98	2.52
	4	6.91	5.14	6.53
0.1	1	1.04	0.96	1.21
	4	4.09	3.14	3.45
0.5	1	0.03	0.01	0.14
	4	2.15	1.12	1.28
1.0	1	0.00	0.00	0.00
	4	0.84	0.00	0.00

TABLE X

Influence of Sodium Oleate on the Hydrolytic Activity of Adrenal Cholesterol Esterase

(Substrate : Inactive human serum, pH 6.9, 38°, Na glycocholate 25 mg.)

Concn. of Na oleate	Time of incubation	Degree of hydrolysis (average values)		
		Expt. I (5 males & 5 females)	Expt. II (5 males & 5 females)	Expt. III (5 males & 5 females)
0.0%	1 *hrs.*	2.15%	1.96%	2.24%
	4	4.24	3.93	4.60
0.01	1	0.72	0.53	1.18
	4	3.01	2.67	3.24
0.05	1	0.00	0.00	0.26
	4	1.32	1.15	2.19
0.1	1	0.00	0.00	0.00
	4	0.00	0.00	1.23
0.5	1	0.00	0.00	0.00
	4	0.00	0.00	0.00

From these tables it is very clear that the activity of the enzyme is enhansed in proportion to the concentration of enzyme and is inhibited in the presence of the products of the reaction.

DISCUSSION

The entire series of reactions reported in the above experiments seem to justify the conclusions that cholesterol esterase exists in the suprarenal body of the rat in both sexes and its esterifying action is remarkably strong than its hydrolytic action. This seems possible that the nature of the enzyme is essentially identical with the one found in other tissues. And it seems to be agreement with the view of Simola (*8*).

The optimum pH lies between 6.8 and 7.0 (6.9). This value is almost the same as in pancreatic cholesterol esterase (6.7~7.1) (*19*). And the optimum pH for hydrolysis is just same as the one for esterifying. But Klein (*2*) reported that the optimum pH of pancreatic cholesterol esterase for esterification is 5.6 and 7.0 for hydrolysis. Nieft (*19*) also observed that the maximum hydrolytic action of serum cholesterol esterase is in 6.4 but the esterifying action is in 5.8. This differnce from Klein's or Nieft's result may be ascribed either to the different experimental condition or to the fundamental differences between adrenal cholesterol esterase and serum or pancreatic cholesterol esterase on this point only.

The results obtained in the activation of adrenal cholesterol esterase by the bile salts are essentially similar to those described for pancreas (*13*), serum (*14*) and intestinal villi (*17*) *etc.* Swell *et al.* (*20*) suggested that the role of bile salts in the activity of cholesterol esterase is in the formation of some type of combination between cholesterol and bile salt. The result of the present experiment may be revealed by this assumption.

It is apparent that the esterifying activity of rat adrenal cholesterol esterase is stronger than its hydrolytic activity in contrary to cholesterol esterase in rat adipose tissue or rat intestinal villi. Generaly the normal suprarenal body is characterized by a high concentration of esterified cholesterol. The results obtained from the above experiments seem to have an interesting bearing on the nature of this fact. It seems possible that the high concentration of esterified cholesterol in suprarenal bodies is due to the existence of the strong esterifying cholesterol esterase system in this organ. The investigation of this possibility must be the subject of future research.

SUMMARY

It is established that the both hydrolytic and esterified cholesterol esterase systems exist in the suprarenal bodies of male and female rats. Its nature is as follows ;

1. The esterifying activity of the enzyme is stronger than its hydrolytic ractivity.

2. The optimum pH of the enzyme stands between 6.8 and 7.0 (6.9).

3. The enzyme is activated by bile salts.

4. Each activity of both enzyme systems is enhanced in proportion to its concentration and is inhibited in the presence of the products of the reaction in proportion to their concentrations.

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STUDIES ON CHOLESTEROL ESTERASE OF THE ORGANS PRODUCING STEROID HORMONES IN THE RAT

II. ITS PRESENCE IN THE TESTES AND OVARY

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It is well known that the organs producing steroid hormones are characterized by a high concentration of esterified cholesterol. In a previous paper of this series a report was made on the existence of cholesterol esterase in the rat suprarenal body, one of the organs producing steroid hormones in mammals (*1*).

In this paper, further observations with the presence of cholesterol esterase in the testes as well as in the ovary of the rat are described, together with some remarks about the experimental method and possible significance of the results.

METHODS AND RESULTS

A. The Presence of Cholesterol Esterase in the Testes

The test animals used in this experiment were Wistar strain adult male rats in body weight 200~250 g. fed with unhulled wheats, green vegetables, salts and water in author's laboratory. The animals were killed by decapitation under ether anaesthesia, the testes (including epididymis) was immeadiately removed after death and homogenized in Kreb's buffer in a homogenizer. Five ml. of the homogenate, equivalent to 20 mg. of tests, was used as enzyme source. The volume of the homogenates was 3.0 ml. The other detailed descriptions of the methods are given by the previous paper.

Influnce of pH—Fig. 1 shows that the optimum pH of the testicular homogenate to esterification of cholesterol lies between 6.6 and 6.9 (6.8). The entirely identical result was obtained in the experiment of testicular homogenate to hydrolysis of esterified cholesterol.

Influence of Activator—From Tables I and II, it is quite evident that testicular cholesterol esterase of the rat is activated by sodium glycocholate. In the control test used sodium glycocholate only any enzymatic action was not observed.

Esterifying Function—Rat testicular cholesterol esterase exerts its esterifying activity both to the incative human serum (Table III) and to the artificial substrate (Table IV).

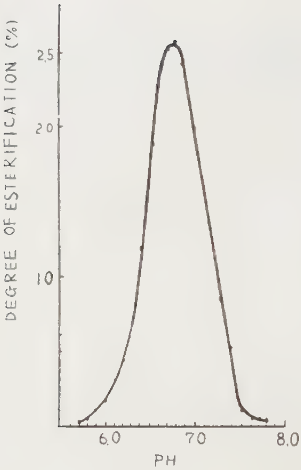

Fig. 1. Esterifying activity of testicular cholesterol esterase under various pH (Substrate : Inactive human serum, 38°, 4 hours incubation).

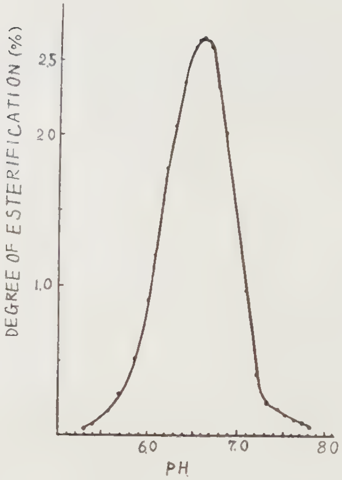

Fig. 2. Esterifying activity of ovarian cholesterol esterase under various pH (Substrate : Inactive human serum, 38°, 4 hours incubation).

Table I

Activation of Esterifying Testicular Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.8, 38°, 4 hours incubation)

Conc. of Na glycocholate in serum	Degree of esterification					
	Rat No. 1211	1212	1213	1214	1215	Average
0‰	1.1%	0.0%	1.9%	1.3%	1.7%	1.20%
10	1.8	1.3	2.2	2.0	2.5	1.96
20	2.3	1.5	3.8	2.9	3.9	2.88
30	2.9	2.2	4.5	3.3	4.8	3.54

But compared with adrenal cholesterol esterase in the previous report its activity is remarkably weak.

Hydrolytic Function—Not only esterifying function but also hydrolytic function of rat testicular cholesterol esterase is observed to the inactive human serum (Table V) and to the artificial substrate (Table V), though its activity is somewhat weak than the

TABLE II

Activation of Hydrolytic Testicular Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.8, 38°, 4 hours incubation)

Concn. of Na glycocholate serum in	Degree of hydrolysis					
	Rat No. 1216	1217	1218	1219	1220	Average
‰	%	%	%	%	%	%
0	0.0	0.1	0.4	0.2	0.3	0.20
10	0.5	0.4	0.6	0.4	0.6	0.50
20	0.8	0.6	1.5	0.5	0.9	0.86
30	1.0	0.9	2.1	0.8	1.2	1.20

TABLE III

Esterification of Inactive Human Serum by Testicular Cholesterol Esterase

(pH 6.8, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of esterification			
	3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
1221	2.1	2.9	3.5	5.8
1222	1.8	2.3	3.1	4.7
1223	1.5	1.6	1.9	2.5
1224	2.5	3.4	4.2	6.1
1225	2.3	3.0	3.3	3.6

TABLE IV

Esterification of Cholesterol witg Sodium Oleate by Testicular Cholesterol Esterase

(pH 6.8, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of esterification			
	3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
1226	1.2	1.5	1.7	2.2
1227	2.4	3.0	4.1	5.6
1228	1.8	2.2	3.6	4.4
1229	2.0	2.8	3.8	5.1
1230	1.1	1.3	1.6	3.0

TABLE V

Hydrolysis of Inactive Human Serum by Testicular Cholesterol Esterase

(pH 6.8, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of hydrolysis			
	3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
1231	0.8	1.2	1.8	2.2
1232	1.2	1.5	1.9	2.5
1233	1.0	1.2	1.3	1.8
1234	1.4	1.8	2.4	3.5
1235	0.9	1.1	1.2	1.3

TABLE VI

Hydrolysis of Cholesteryl Oleate Emulsion by Testicular Cholesterol Esterase

(pH 6.8, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of hydrolysis			
	3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
1236	0.0	0.2	0.3	0.8
1237	0.2	0.5	0.9	1.2
1238	0.4	0.6	0.6	1.1
1239	0.6	0.9	1.2	1.8
1240	0.5	0.6	0.8	1.3

TABLE VII

Influence of the Quantity of Testicular Cholesterol Esterase on its Esterifying Activity

(pH 6.8, 38°, Na glycocholate 25 mg.)

Rat No.	Quantity of testicular homogenate		Esterification after 4 hrs. incubation (average values)
	ml.		%
1242, 1241, 1243	0		0
1241, 1242, 1243	1		1.06
1241, 1242, 1243	2		2.61
1242, 1243, 1244	5		6.35
1241, 1242, 1245	10		11.42
1241, 1243, 1245	5	boiled at 60°	0
1241, 1242, 1244	10	for 20 minutes	0

Table VIII

Influence of the Quantity of Testicular Cholesterol Esterase on Its Hydrolytic Activity

(pH 6.8, 38°, Na glycocholate 25 mg.)

Rat No.	Quantity of testicular homogenate homogenate	Hydrolysis after 4 hrs. incubation (average values)
	ml.	%
1246, 1247, 1248	0	0
1246, 1247, 1248	1	0.85
1247, 1248, 1249	2	1.18
1246, 1248, 1249	5	2.24
1246, 1247, 1250	10	4.67
1246, 1249, 1250	5 boiled at 60°	0
1248, 1249, 1250	10 for 20 minutes	0

Table IX

Influence of Cholesteryl Oleate on the Esterifying Activity of Testicular Cholesterol Esterase

(pH 6.8, 38°, Substrate : Inactive human serum, Na glycocholate 25 mg.)

Concn. of cholesteryl oleate	Time of incubation	Degree of esterification (average values)	
		Expt. I (5 males)	Expt. II (5 males)
%	*hrs.*	%	%
0.0	1	1.83	1.61
	4	2.54	2.28
0.01	1	0.92	1.08
	4	1.87	1.44
0.05	1	0.07	0.21
	4	0.89	0.74
0.1	1	0.00	0.00
	4	0.00	0.00

esterifying activity.

Influence of the Concentration of the Enzyme—That the activity of testicular cholesterol esterase of the rat increases in proportion to its concentration is indicated in Tables VII and VIII.

TABLE X

Influence of Sodium Oleate on the Hydrolytic Activity of Testicular Cholesterol Esterase

(pH 6.8, 38°, Substrate : Inactive human serum, Na glycocholate 25 mg.)

Concn. of sodium oleate	Time of incubation	Degree of hydrolysis (average values) Expt. I (5 males)	Degree of hydrolysis (average values) Expt. II (5 males)
%	*hrs.*	%	%
0.0	1	0.81	0.84
	4	1.29	1.37
0.01	1	0.21	0.28
	4	0.62	0.75
0.05	1	0.00	0.00
	4	0.17	0.11
0.10	1	0.00	0.00
	4	0.00	0.00

And it is shown in Tables IX and X that the activity of the enzyme is inhibited in the presence of the products of the reaction.

B. *The Presence of Cholesterol Esterase in the Ofvary*

The test animals used were Wistar strain adult female rats in body weight 180~230 g. fed with unhulled wheats, green vegetables, salts and water in author's laboratory. As the enzyme source 5 ml. of the homogenate, equivalent to 5 mg. of the ovary, was used. The volume of the homogenate used in this experiment was 3.0 ml. The other detailed description of the methods is given by the previous paper.

The results of the experiments are shown in the following figure (Fig. 2) and tables (Tables XI~XX). The figure and these tables summarize the results as follows : 1) the ovary of the rat has both hydrolytic and esterifying cholesterol esterase systems ; 2) the optimum pH of the enzyme lies between 6.5 and 6.8 (6.6) ; 3) the esterifying activity of this enzyme is stronger than its hydrolytic activity ; 4) the activity of the enzyme is activated by sodium glycocholate and 5) the activity of the enzyme increases in proportion to its concentration and retards in the presence of the products of its reaction.

DISCUSSION

The results of the present study indicate that there exist both esterifying and hydrolytic cholesterol esterase systems in the rat testes and ovary, though each activity is considerably weak as compared with the adrenal cholesterol esterase systems reported in the previous paper

TABLE XI

Activation of Esterifying Ovarian Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.6, 38°, 4 hours incubation)

Concn. of Na glycocholate in serum		Degree of esterification					
	Rat No.	6521	6522	6523	6524	6525	Average
‰		%	%	%	%	%	%
0		0.9	0.2	1.2	1.8	1.5	1.12
10		1.8	1.2	2.1	3.9	1.9	2.18
20		2.6	1.8	3.6	4.7	2.1	2.96
30		3.5	2.4	4.3	5.2	2.6	3.60

TABLE XII

Activation of Hydrolytic Ovarian Cholesterol Esterase by Various Concentrations of Sodium Glycocholate

(Substrate : Inactive human serum, pH 6.6, 38°, 4 hours incubation)

Concn. of Na glycocholate in serum		Degree of esterification					
	Rat No.	6526	6527	6528	6529	6530	Average
‰		%	%	%	%	%	%
0		0.6	1.0	2.0	0.4	0.6	0.92
10		0.9	1.8	2.5	0.6	0.7	1.20
20		1.4	2.2	2.8	0.9	1.0	1.66
30		1.6	2.5	3.0	1.1	1.2	1.88

TABLE XIII

Esterification of Inactive Human Serum by Ovarian Cholesterol Esterase

(pH 6.6, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of esterification			
	3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
6531	2.3	2.9	3.5	5.0
6532	2.5	3.2	3.8	5.1
6533	1.8	2.5	3.0	3.8
6534	2.4	3.0	4.2	6.8
6535	1.6	1.9	2.2	2.7

TABLE XIV

Esterification of Cholesterol with Sodium Oleate by Ovarian Cholesterol Esterase

(pH 6.6, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of esterification 3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
6536	1.9	2.5	3.3	5.8
6537	1.6	2.0	3.1	4.4
6538	2.8	3.4	4.0	6.2
6539	2.2	3.0	3.8	4.9
6540	1.4	1.6	1.8	2.5

TABLE XV

Hydrolysis of Inactive Human Serum by Ovarian Cholesterol Esterase

(pH 6.6, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of hydrolysis 3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
6541	1.7	1.8	2.0	3.7
6542	1.0	1.4	1.5	2.9
6543	0.0	0.2	0.3	1.2
6544	2.1	2.7	3.3	4.5
6545	1.5	1.8	2.2	3.4

TABLE XVI

Hydrolysis of Cholesteryl Oleate Emulsion by Ovarian Cholesterol Esterase

(pH 6.6, 38°, Na glycocholate 30 mg.)

Rat No.	Degree of hydrolysis 3 hrs.	6 hrs.	12 hrs.	24 hrs.
	%	%	%	%
6546	1.0	1.1	1.5	2.2
6547	1.4	1.5	1.6	2.8
6548	0.1	0.4	0.5	0.8
6549	0.0	0.1	0.3	0.6
6550	0.9	1.1	1.7	2.1

TABLE XVII

Influence of the Quantity of Ovarian Cholesterol Esterase on Its Esterifying Activity

(pH 6.6, 38°, Na glycocholate 25 mg.)

Rat No.	Quantity of ovarian homogenate	Esterification after 4 hrs. incubation (average values)
	ml.	%
6551, 6552, 6553	0	0
6552, 6553, 6554	1	1.46
6552, 6553, 6554	2	2.72
6551, 6553, 6554	5	7.23
6551, 6552, 6555	10	13.91
6551, 6554, 6555	5 boiled at 60°	0
6551, 6552, 6553	10 for 20 minutes	0

TABLE XVIII

Influence of the Quantity of Ovarian Cholesterol Esterase on Its Hydrolytic Activity

(pH 6.6, 38°, Na glycocholate 25 mg.)

Rat No.	Quantity of ovarian homogenate	Hydrolysis after 4 hours incubation (averate values)
	ml.	%
6556, 6557, 6558	0	0
6556, 6558 6559	1	0.73
6557, 6558, 6559	2	1.37
6556, 6557, 6559	5	2.54
6556, 6557, 6560	10	4.96
6556, 6557, 6560	5 boiled at 60°	0
6556, 6558, 6560	10 for 20 minutes	0

(*1*). So far as is known, the work reported here is the first specifically designed to elucidate the presence of cholseterol esterase in the testes and in the ovary. But it is assumed that since there is a considerable degree of correspondence between the results obtained with the optimum pH, the behaviour of the action and activation by bile salts, and the experiment carried out on the same points in adrenals (*1*), pancreas (*2*) or in

TABLE XIX

Influence of Cholesteryl Oleate on the Esterifying Activity of Ovarian Cholesterol Esterase

(pH 6.6, 38°, Substrate : Inactive human serum, Na glycocholate 25 mg.)

Concn. of cholesteryl oleate	Time of incubation tion	Degree of esterification (average values) Expt. I (5 females)	Expt. II (5 females)
%	hrs.	%	%
0.0	1	2.02	1.81
	4	2.86	2.45
0.01	1	0.80	0.62
	4	1.25	1.33
0.05	1	0.00	0.00
	4	0.44	0.38
0.1	1	0.00	0.00
	4	0.00	0.00

TABLE XX

Influence of Sodium Oleate on the Hydrolytic Activity of Ovarian Cholesterol Eesterase

(pH 6.6, 38°, Substrate : Inactive human serum, Na glycocholate 25 mg.)

Concn. of sodium oleate	Time of incubation	Degree of hydrolysis (average values) Expt. I (5 females)	Expt. II (5 females)
%	hrs.	%	%
0.0	1	0.65	0.84
	4	1.18	1.39
0.01	1	0.21	0.33
	4	0.53	0.76
0.05	1	0.00	0.00
	4	0.25	0.38
0.1	1	0.00	0.00
	4	0.00	0.00

intestinal villi (*3*), cholesterol esterase in the rat testes and ovary is essentially similar to those described already for these other organs.

The testes and the ovary are esterified cholesterol rich organs. It seems to be very interesting that the esterifying system of cholesterol esterase with high activity exists in these organs. Though Claesson *et al.* (*4*, *5*) have assumed that esterified cholesterol is mobilized with ease within the ovary of the rat, there seems to be little evidence to support their assumption. Because from the results of author's experiment it seems very improbable that the activity of esterified ovarian cholesterol esterase system is not high enough to be able to mobilize esterified cholesterol so easily. It may be suggested that cholesterol esterase in these reproductive organs, including supra-renal body, plays some role in the formation of steroid hormones, but these points are being investigated.

SUMMARY

From this series of experiments, it may be concluded:

1. Both hydrolytic and esterifying cholesterol esterase systems are established in the testes and the ovary of the rat, though their activity is remarkably weak as compared with adrenal cholesterol esterase. The differences of the enzyme activity are not almost recognized between the testes and the ovary.
2. Esterifying activity of these two enzymes are stronger than their hydrolytic activity.
3. The optimum pH of the enzymes are 6.8 (6.6~6.9) in the testes and 6.6 (6.5~6.8) in the ovary.
4. These two enzymes are activated by bile salts.

The author wishes to express his thanks to Prof. Raijiro Takahashi for his interest and encouragement. Thanks are ablso due to Mr. Mitsuyoshi Ueno, Mr. Atsushi Irizawa and Mr. Shigeki Umezane for their able collaborations in the course of these experiments. And this work has been partly supported by a grant in aid for the Miscellaneous Scientific Research from the Ministry of Education in 1953.

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STUDIES ON METAPHOSPHATE

II. HEAT OF HYDROLYSIS OF METAPHOSPHATE EXTRACTED FROM YEAST CELLS

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In the previous papers, it has been elucidated that the formation of metaphosphate from orthophosphate by intact yeast cells or extracted enzyme system is coupled with energy metabolism, and ATP may be an intermediate product of the process. (*1*) It has also been recognized that metaphosphate and ADP are reversibly converted to ATP. (*2*) These facts suggest that metaphosphate contains " energy rich phosphate bonds ".

O. Meyerhof *et al.* (*3*) using a microcalorimetric apparatus, determined the heat of hydrolysis of trimetaphosphate and estimated it to be about 19,000 cal. per mol. However, metaphosphate extracted from yeast cells is different in several characteristics from trimetaphosphate ; for example, while the former shows metachromatic reaction with toluidine blue and is well precipitated with proteines, the latter does not. Yeast metaphosphate shows properties similar to those of "Graham's salt ", and its molecular weight may be considerably large. So it is desirable to determine the P-O-P bond energy of yeast metaphosphate and " Graham's salt ".

The author extracted from yeast an enzyme solution which catalyses the reversible synthesis of ATP from metaphosphate and ADP (*2*), and attempted to obtain the free energy of hydrolysis of metaphosphate from the equiliblium constant of this reaction. But this attempt proved unsuccessful, because the enzyme solution contained metaphosphatase, ATPase and adenylate-kinase, and the side reactions by these enzymes could not be eliminated. Therefore, direct measurements of the eat evolved by the enzymic hydrolysis of yeast metaphosphate and " Graham's salt " were carried out with an isothermal constant flow calorimter.

EXPERIMENTAL METHODS

Calorimeter—The apparatus used was simillar to that of Ohlmeyer's in principle (*4*), but was different from the latter in several points. A diagram of the apparatus is shown in Fig. 1. A thermister instead of a mercury regulator, was used as a regulator of the reaction vessel. Owing to the smallness of the heat capacity of the thermister regulator, the sensitivity of the apparatus was higher than that using a mercury regulator. The temperature inside the reaction vessel (Tv) and that of the outer bath (Tb) were kept constant by servomechanisms controlling electric heaters. As Tv was a little higher than Tb, a balance between the input and output was required to keep Tv constant. Before the reaction, the output was equal to the heat provided by an electric heater. After the reaction, the heat supply might become smaller by the effect of heat of reaction. Heating time was measured by a gas coulometer connected to the relay.

The reaction heat in gram calories is given by the expression :

$$\Delta H = R \times i \times \frac{\Delta V}{0.6264} \times \frac{3.6}{4.185}$$

where

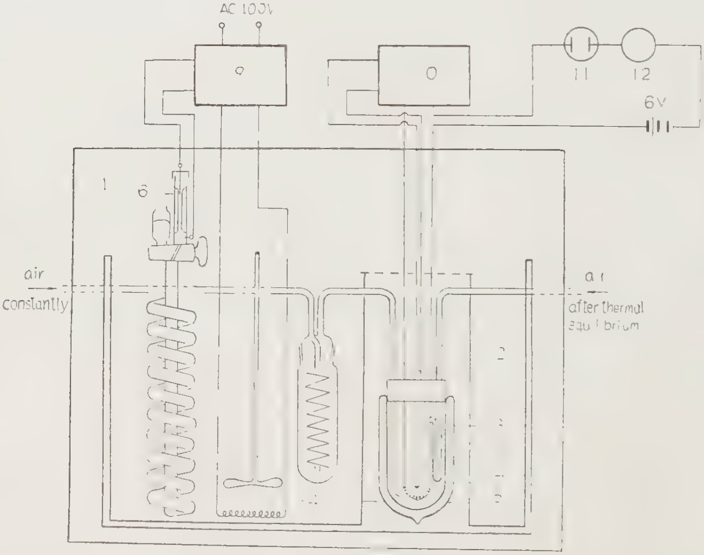


FIG. 1 A. Isothermal constant flow calorimeter

1. Incubator 2. Outer bath 3. Reaction vessel 4. Heater of outer bath 5. Heater of reaction vessel 6. Mercury regulator 7. Thermister 8. Enzyme vessel 9. Electric relay 10. Thermister relay (Fig. 1 B) 11. Gas coulometer 12. Milliammeter

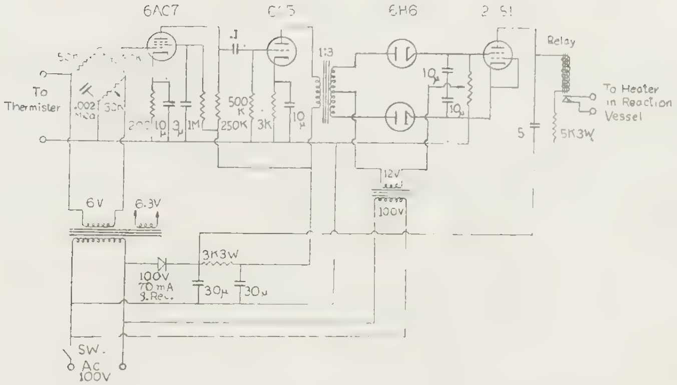


FIG. 1 B. Circuit of Thermister Relay

$$\varDelta V = V_0 \times \frac{t}{t_0} - V$$

V_0 : Gas evolved during t_0 hours before the reaction (in ml. at 0° and 1 atm.).
V : Gas evolved during t hours after the reaction (in ml. at 0° and 1 atm.).
R : Resistance of heater inside the reaction vessel (in ohm).
i : Current of heater inside the reaction vessel (in amp.).

The increase of gas volume was measured every 10 or 15 minutes. After the gas volume reached a steady state, i.e. the thermal equilibrium was attained, enzyme solution was mixed with substrate solution.

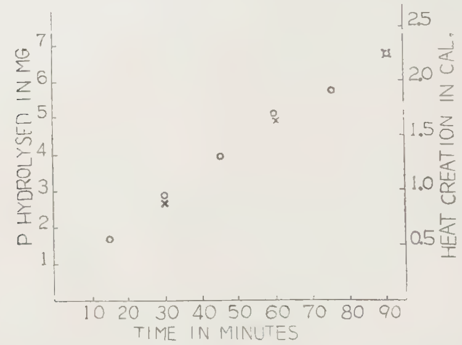
Preparation of Enzyme—The enzyme was extracted from rice brain and purified by fractional precipitation with acetone (*5*). About 2 per cent solution of the enzyme power in $5 \times 10^{-2} M$ acetate buffer solution was used as the enzyme solution.

Preparation of Yeast Metaphosphate and "Graham's salt"—Phosphate-starved yeast (*1*) (10 gr. in wet weight) was suspended in 250 ml. of a medium containing $2 \times 10^{-2} M$ $MgSO_4$, $2 \times 10^{-2} M$ $(NH_4)_2SO_4$, $5 \times 10^{-3} M$ KCl, $10^{-2} M$ K_2HPO_4, 1 per cent glucose and $2 \times 10^{-2} M$ succinate buffer of pH 5.2, and aerated for 2 hours. After this treatment, metaphosphate content of yeast was about 20 mg. phosphorus per 1 g. of dry yeast. Metaphosphate-rich yeast cells were washed three times with 200 ml. of water, and extracted three times with 50 ml. of 5 per cent solution of ice cold trichloroaceticacid. After washing twice with water, the yeast cells were defatted with alcohol-ether and extracted with water at pH 9.0. Metaphosphate was precipitated as Ba salt at pH 4.0 from the extracted solution. The precipitate was dissolved in an adequate quantity of $10^{-2} N$ HCl and then Na_2SO_4 was added to eliminate Ba. After centrifugation, the supernatant was neutralized to pH 7.0. "Graham's salt" was prepared in the usual way from NaH_2PO_4 by heating.

EXPERIMENTAL RESULTS

The course of heat evolution and the appearance of orthophosphate are shown in Fig. 2. It is evident that the heat evolution was accompanied by hydrolysis of metaphosphate. From this experiment, it can be calculated that the heat of hydrolysis per P-O-P bond is about 10,000 cal. Heat of hydrolysis of yeast metaphosphate, "Graham's salt" and pyrophosphate are shown in Table I.

Fig. 2. Heat Creation and Metaphosphate Hydrolysis
×......Metaphosphate Hydrolysed in mg. P
○......Heat Creation in cal.



The reaction mixture contained yeast metaphosphate contained 23 mg. P, $5\times10^{-2}M$ acetate buffer at pH 5.0 and enzyme solution. Total volume 52 ml.

Heat of hydrolysis of yeast metaphosphate is about 10,000 cal. per P-O-P bond at pH 5.0, and that of "Graham's salt" is 11,000 cal. at the same pH. Considering the ionization states of reactants and products at 30° and pH 5.0 (at which pH value the heat of neutrarization is negrigible), the decrease of enthalpy of yeast metaphosphate is estimated to be 9,700±500 cal. per phosphate bond, and that of "Graham's salt" to be 11,000±500 cal.

DISCUSSION

Free energy change of hydrolysis (ΔF) is equal to $\Delta H - T\Delta S$, where ΔS is the entropy change of hydrolysis. Molecular entropy is in general the sum of rotational entropy, vibrational entropy and transrational

TABLE I

Heat of Hydrolysis of Metaphosphate

	pH	heater current mamp	heater resistance ohm	ΔV ml	Phosphate hydrolysed P mg	ΔH/P-O-P cal
Yeast Metaphosphate	5.0	7.2	122	0.42	1.58	10,000
	5.0	7.1	122	0.52	2.23	8,600
	5.0	7.7	115.7	1.58	7.12	9,800
Graham's salt	5.0	6.5	122	1.83	5.49	11,700
	5.0	7.0	122	1.73	6.20	10,100
	5.0	9.4	122	0.91	5.89	11,600
	5.0	8.2	115.7	0.306	1.13	10,700
	6.0	7.4	122	1.34	4.36	9,800
Pyrophosphate	5.0	9.1	122	0.49	4.66	10,000

The substrate solution contained yeast metaphosphate or "Graham's salt contained $3\sim23$ mg. P and $5\times10^{-2}M$ acetate buffer. Total volume was 50 or 30 ml.

After thermal equilibrium was attained, 2 or 1.5 ml. of enzyme solution was mixed with substrate solution.

Experimental error was considered to be ±500 cal.

entropy of the molecule. Among these three entropies, rotational entropy may be greatly changed by hydrolysis ; because the degree of freedom of rotation of atomic groups would increase as a result of hydrolysis. In trimetaphosphate molecule, which has a ring structure, rotation of phosphate groups are inhibited. The hydrolysis will render the rotation of phosphate free and, therefore the entropy greater. According to the experimental results of Meyerhof et *al.* the heat of hydrolysis of trimetaphosphate is about 6,300 cal. per P-O-P bond. The free energy change of hydrolysis must be more or less greater than 6,300 cal. It is difficult to evaluate the entropy change of hydrolysis exactly, but judging from the data of other molecules containing P-O-P bond, the free energy change of hydrolysis may be about $10{,}000\sim12{,}000$ cal. per P-O-P bond.

Heat of hydrolysis of inorganic pyrophosphate, which has a chain structure, is about 9,000 cal. at pH 7.0. In this case, since the rotation around the P-O-P bond is not restricted completely, the increase of entropy owing to hydrolysis may not be so great as in the case of trimetaphosphate. Yeast metaphosphate and "Graham's salt", which are recognized as considerably large chain molecules, may show small entropy change in the process of hydrolysis. It may be considered that the free energy change of hydrolysis of P-O-P bond would be of the same order of magnitude in various compounds containing the P-O-P bond. The difference in the heat of hydrolysis of the bond in different phosphorous compounds might be attributed to the difference of entropy change in the process of hydrolysis.

In conclusion the author should like to thank Prof. T. Soda for his valuable guidance, and Mrs. K. Sakata for her valuable advice conserning thermister regurator.

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A NEW METHOD OF CHROMATOGRAPHIC DIFFERENTIAL MICRODETERMINATION OF BILE ACIDS IN BILE

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Silberman (*1*) and Griesmann and Falck (*2*) reported the application of chromatography to the determination of bile acids. Differential determination of bile acids in ox bile by counter current distribution method reported by Ahrens and Craig (*3*) requires a special equipment. Paper chromatography (*4*, *5*) seems to be inadequate for our purpose as a method of quantitative analysis. The application of column partition chromatography reported recently by Bergström and Sjövall (*6*), Norman (*7*), and Sjövall (*8*) have been proved to be an excellect method for isolation and quantitative analysis of each type of bile acids.

Murakami (*9*) modified the methods of Abe (*10*) and Kawaguchi (*11*), and devised a new method for the differential determination of free and conjugated bile acids in bile, utilizing the difference of their solubility in ether. But in this method, the quantity of free bile acid is determinable only indirectly from that of conjugated acid by calculation, and so the minute quantity of free bile acids in bile is practically indeterminable, as will be explained later.

The present paper deals with the results of the author's experiment, which was commenced for the purpose of devising a method for speedy and accurate differential microdetermination of bile acids by liquid chromatography. The experiment succeeded in separating free bile acids from conjugated ones though not yet in separating individual bile acids each other.

Each of the isolated bile acid could, however, be determined colorimetrically by Murakami's method (*9*), so that the differential determination of tri-and di-hydroxycholanic acids in both free and conjugated types were made possible, though desoxycholic and chenodesoxycholic acids could only be determined inseparably in this experiment.

Besides, glycine and taurine-conjugated trihydroxycholanic acids on the one hand, and glycine and taurine-conjugated dihydroxycholanic acids on the other were each determined as one substance, as is the case in Marakami's method.

EXPERIMENTAL

Chromatographic Differential Determination of Free and Conjugated Bile Acids

Procedures for Quantitative Analysis—

Reagents—

1) *Adsorbent:* Activated alumina, 200 mesh, Wako.
2) *A 10 per cent acetic acid alcohol solution:* A mixture of 90 parts of absolute alcohol and 10 parts of pure glacial acetic acid.
3) *Phosphoric acid I:* Sp. gr. 1.625 at 22°.
4) *Phosphoric acid II:* Sp. gr. 1.750 at 22°.
5) *Vanillin phosphoric acid solution I:* 1.0 mg. of pure vanillin is completely dissolved in 1.5 ml. of phosphoric acid I.
6) *Vanillin phosphoric acid solution II:* 1.0 mg. of pure vanillin is completely dissolved in 1.5 ml. of phosphoric acid II.
7) *Walpole's acetate buffer.* pH 4.0.
8) *Ether:* redistilled and saturated with Walpole's acetate buffer (pH 4.0).
9) *A 5 per cent acetic acid alcohol solution:* A mixture of 5 parts of pure glacial acetic acid and 95 parts of absolute alcohol.

Preparation of Column—To just 5 g. of activated alumina in a small Erlenmeyer flask 10 ml. of 10 per cent acetic acid alcohol solution was added gently under stirring, and left standing at room temperature for about 30 minutes. A greater part of the supernatant layer was discarded and alumin was poured through a funnel while being agitated into the chromatographic tube (int. diam. 7∼8 mm. hight about 300 mm.). Alumina in the tube was washed several times with 10 ml. of absolute alcohol in divided portions. The sample was then poured in it for adsorption.

Addition and Development of Sample to Column—

1) Preparation of Sample: Into a definite amount of bile in a flask several times as much 96 per cent alcohol was added and heated at 80∼90° for 2∼3 minutes on a water-bath, and then left standing at room temperature. The cooled solution was filtered and a clear transparent proteinfree solution was obtained. The residure was washed several times with small quantity of alcohol, and all the abluents were poured into the filtrate, which was left standing overnight, if clear enough, it was filled up with alcohol to the volume sufficient for experiment. When turbid and sedimental, the filtrate was again heated for 2∼3 minutes, after being cooled, filtered off until completely cleared, and filled up to the definite volume.

Firstly, the total bile acids content (total tri- and di-hydroxycholanic acids) of this filtrate was determined colorimetrically by the Murakami's method (*9*).

Next, another definite portion of the filtrate was taken into a small Erlenmeyer flask and alcohol was evaporated up under reduced pressure. To the dry residues 4.0 ml. of Walpole's acetate buffer (pH 4.0) was added. Bile acid was extracted 4 times with 5 ml. of ether saturated with the buffer solution. By this procedure free bile acid was almost entirely, and conjugated bile acid partially, brought into the ether. The ether layer was completely dried on a water-bath, and the residue was dissolved in 5 ml. of absolute alcohol for further analysis.

2) Addition, Adsorption and Development of Sample: Immediately after the preparation of the above adsorption column, 5 ml. of the sample solution was poured into the column completely, and the column was washed with 10 ml. of absolute alcohol for the development of bile acid adsorbed.

Elution, Collection of Effluent Fraction and Its Analysis—As the eluent for the differential elution of free and conjugated bile acids from the column, 5 per cent acetic acid alcohol solution was used. This eluent was poured into the column and the bile acids were left to drift down spontaneously to be eluted. The first 10 ml. of the effluent contained 80±2 per cent of the total amount of free bile acids (tri- and di-hydroxycholanic acids) used. The subsequent effluent of a minute amount of free bile acids was not taken into calculation in this experiment. As conjugated bile acid was always absent in the first 14 ml., the first 10 ml. was a sample completely free from the conjugated acids. Then, an adequate volume was taken from the first 10 ml. into two 50 ml. Erlenmeyer flasks. If any turbidity occurred on adding vanillin phosphoric acid, 5 ml. of the remaining effluent was shaken with petroleum ether for removal of the substances causing turbidity. To put it in more detail, 5 ml. of the effluent in a Erlenmeyer flask was evaporated to dryness under reduced pressure, to which 1 ml. of 0.3∼0.4 per cent HCl was added (pH 2.0∼3.0) to precipitate bile acids, and 10 ml. of petroleum ether was added, shaken well, and filtered. The precipitate of bile acids on the filter paper was washed well with petroleum ether and dissolved in 96 per cent alcohol perfectly and then filled up to a definite volume for colorimetric analysis. There was no detectable loss of bile acids by this treatment.

Calculation—The quantities of trihydroxycholanic acid and dihydroxycholanic acids of free and conjugated types are known from the quantity of total bile acids determined (all of the trihydroxycholanic and dihydroxycholanic acids of free and conjugated types) and that of total free bile acids determined after chromatographic procedure (free trihydroxycholanic and all free dihydroxycholanic acids). The amount of bile acids is given from the following formula, as Murakami calculated (*9*) :

$E_{1\,\mathrm{tri}}$: extinction-coefficient for total trihydroxycholanic acids obtained with vanillin phosphoric acid solution I.

$E_{1\,\mathrm{mix}}$: extinction-coefficient for total trihydroxycholanic plus total dihydroxycholanic acids obtained with vanillin phosphoric acid solution II.

$E_{c\,\mathrm{tri}}$: extinction-coefficient for trihydroxycholanic acid of free type obtained with vanillin phosphoric acid solution I after chromatographic procedure.

$E_{c\ mix}$: extinction-coefficient for trihydroxycholanic plus dihydroxycholanic acids of free type obtained with vanillin phosphoric acid solution II after chromatographic procedure.

(Those $E_{1\ tri}$, $E_{1\ mix}$, $E_{c\ tri}$, and $E_{c\ mix}$. are obtained from the same dilution of the original bile.)

Amount of trihydroxycholanic acid of free type

$$=\frac{E_{c\ tri}}{0.1417}\times\frac{100}{80}\times(\text{times of dilution})\ \text{mg./ml. of original bile}$$

Amount of dihydroxycholanic acid of free type

$$=0.1\times\frac{E_{c\ mix}-(E_{c\ tri}\times0.89)}{0.216}\times\frac{100}{80}\times(\text{times of dilution})\ \text{mg./ml. of original bile}$$

Amount of trihydroxycholanic acid of conjugated type

$$=0.1\times\frac{E_{1\ tri}-(E_{c\ tri}\times\frac{100}{80})}{0.1277}\times(\text{times of dilution})\ \text{mg./ml. of original bile}$$

Amount of dihydroxycholanic acid of conjugated type

$$=0.1\times\frac{E_{1\ mix}-(E_{c\ mix}\times\frac{100}{80})-0.89\times(E_{1\ tri}-E_{c\ tri}\times\frac{100}{80})}{0.2}\times(\text{times of dilution})$$

mg./ml. of original bile

Selection of Optimal Conditions

Adsorbent—The adsorbent employed for the separation of bile acid was activated alumina :

TABLE I

Adsorption of Pure Na cholate by Acetic Acid-treated Alumina

Alumina (chosen for treatment with acetic acid)		Merk Brockmann	Wako 200 mesh
Amount of alumina used	g.	5.0	5.0
Amount of sample	mg.	8.0	8.0
Recovery of sample after elution	%	92.5	93.1

Merk's alminium oxide (standardized for chromatographic adsorption analysis acc. to Brockmann) and Wako's activated alumina (purified products, 200 mesh) were compared with each other (See Table I).

Treatment of alumina with 10 per cent acetic acid alcohol raised the recovery rate as shown in Table II.

Solvent—Alcohol was used as solvent because it never interferes with the coloring

TABLE II

Activation of Alumina

(Experiment with 4.4 mg. of pure Na cholate)

Collection of effluent	Bile acid eluted	
	Alumina treated with 10% acetic alcohol	Alumina nontreated
1st. 5 *ml.*	0.0 %	0.0 %
2nd. 5 "	82.0	46.8
3rd. 5 "	7.8	16.3
4th. 5 "	2.8	7.3
5th 5 "	trace	5.1
Total	92.7	75.5

reaction. Asbolute alcohol was preferable because any water content gave a delicate effect on the chromatography. As shown in Table III, the recovery was greater with absolute alcohol than that containing water.

TABLE III

(Experiment with 3.5 mg. of pure Na cholate)

Collection of effluent	Bile acid eluted	
	Absolute alcohol	95% alcohol
1st. 5 *ml.*	0.0 %	0.0 %
2nd. 5 "	81.5	64.0
3rd. 5 "	8.7	8.9
4th 5 "	2.0	2.1
Total	92.2	75.0

Eluent—The 5 per cent acetic acid alcohol was used. The addition of acetic acids to alcohol was found to accelerate the elution of bile acids, as shown in Table IV.

As shown in Table V, the first 10 ml. of eluate contained 82.3 per cent of free bile acids used but no conjugated bile acid. The latter did not appear until the eluates

TABLE IV

(Experiment with 5.3 mg. of Na cholate)

Collection of effluent	Bile acid eluted				
	Alcohol	Alcohol+ acetic acid (1%)	Alcohol+ acetic acid (3%)	Alcohol+ acetic acid (5%)	Alcohol+ acetic acid (10%)
1st. 5 *ml.*	0 %	0 %	0 %	0 %	0 %
2nd. 5 "	0	0	69.6	82.3	85.0
3rd. 5 "	0	58.0	17.0	8.0	5.5
4th 5 "	0	19.2	5.4	2.7	2.0
5th 5 "	0	6.5	trace	trace	trace
6th 5 "	0	4.0	0	0	0

reached to 14 ml. Therefore by taking the first 10 ml. the two types of bile acids were determinable differentially. Since the first 15 ml. of the eluates, which 90 per cent of free bile acids were found, might contain some conjugated acids, only the first 10 ml. were used for colorimetric analysis.

TABLE V

	Collection of effluent	5% acetic acid alcohol		10% acetic acid alcohol	
		Free cholate 5.3 mg.	Conjugated glycocholate+glycodesoxycholate 7.8 mg.	Free cholate 5.3 mg.	Conjugated glycocholate+glycodesoxycholate 7.8 mg.
Percent of eluted bile acid	1st. 5 *ml.*	0 %	0 %	0 %	0 %
	2nd. 5 "	82.3	0	85.0	8.7
	3rd. 5 "	8.0	trace	5.5	48.3
	4th 5 "	2.7	5.5	2.0	19.5
	5th 5 "	trace	19.6	trace	5.6
	6th 5 "	0	25.4	0	3.8

As shown in Table V, the amount of bile acids eluted in the first 10 ml. was 85 per cent when 10 per cent acetic acid alcohol was used.

But this eluent was less appropriate for the differentiation of free and conjugated bile acids compared with 5 per cent acetic acid alcohol. The addition of acetone, ether, chloroform, or petroleum ether to this eluate did not improve the separation and

TABLE VI

Experiment with Pure Bile Acid

Na salt of bile acid	Amount of bile acid added	Amount of bile acid found in 1st 10 ml. of eluates	
Cholate	0.8 *mg.*	0.65 *mg.*	81.3 %
"	2.57	2.04	79.7
"	3.0	2.42	80.7
"	3.7	2.98	80.7
"	4.5	3.54	78.7
"	5.27	4.34	82.3
"	7.8	6.35	81.4
			average 80.7
Desoxycholate	1.1	0.89	80.9
"	1.32	1.05	79.7
"	1.9	1.56	82.2
"	2.17	1.72	79.5
"	3.8	3.09	81.5
"	5.4	4.32	80.1
Chenodesoxycholate	1.8	1.44	79.8
"	3.55	2.85	80.3
Dihydroxycholanate			average 80.5
Cholate	2.3	1.85	80.5
+	+	+	+
desoxycholate	2.5	2.03	81.2

recovery rates to any perceptible extent. With the use of 5 per cent acetic acid alcohol the recoveries in each first 10 ml. were 80±2 per cent in cases of trihydroxy- and dihydroxy-cholanic acid, respectively (Table VI).

As shown in Table VII, the recovery of trihydroxy-and dihydroxy-cholanic acid was nearly the same—79.4±1 and 79.4±1 per cent, respectively, when they were added to bile as pure free acids.

These results showed that the free bile acids recovered in the first 10 ml. eluted by this method was 80 per cent within the error of 5 per cent.

As ascertained in recovery tests of bile acid, the recovery of free bile acid corrected was ca. 99 per cent for the amount originally used, proving the accuracy of this method for quantitiatve determination.

Preparation of Sample—It is not available in this method to use the bile in its original concentrated state. It is appropriate to use an amount containing 20∼50 mg. of total bile acids.

The protein in bile gives red-color by the vanillin phosphoric acid reagent and interferes with chromatography. The removal of protein from bile should be made completely by the procedure described above. The water had to be eliminated completely ; so the protein-free alcohol solution of bile acids was evaporated to dryness and dissolved in absolute alcohol. This alcohol solution contained some turbid substances, which

TABLE VII

Recovery Test of Pure Free Bile Acids Added to Human Bile

Bile				Free bile acid added		Free bile acid recovered	
Patient	Disease	Volume	Free bile acid content	Kind	Amount	Amount in 1st 10 ml. of eluates	Per cent in 1st 10 ml. of eluates to total
		ml.	*mg.*		*mg.*	*mg.*	%
Y.U.	*Ulcus ventriculi*	0.5	0	Cholate	1.5	1.19	79.3
		0.5	0	Desoxycholate	1.9	1.52	80.0
T.K.	*Gastritis chron.*	1.0	0	Cholate	3.17	2.53	80.0
		1.0	0	Desoxycholate	1.32	1.05	79.5
Y.A.	*Ulcus ventriculi*	0.5	0	Cholate	0.78	0.61	78.2
		0.5	0	Desoxycholate	2.17	1.71	78.8
H.K.	„	0.5	0	Cholate	8.4	6.67	79.4
		0.5	0	Desoxycholate	1.9	1.53	80.5
		0.5	0	Chenodesoxycholate	3.55	2.79	78.5
G.S.	*Cholelithiasis*	4.0	1.8	Cholate	4.8	5.25	79.5

interfered with the colorimetric determination by using vanillin phosphoric acid reagents as shown in Table VIII.

TABLE VIII

Differential Analysis Made without Ether Treatment

Bile		Free bile acid		
Amount used	Free bile acid content	Added		Found
ml.	*mg.*		*mg.*	*mg.*
0.5	0	Cholate	3.5	4.0
0.5	0	Desoxycholate	2.0	3.0

Accordingly, a definite quantity of this alcohol solution was taken and, after dryness, the residues were dissolved in Walpole's buffer (pH 4.0). From this buffer solution all of the free bile acids moreover were extracted with ether.

By this treatment the turbid substances were completly excluded. The ether solution was evaporated to dryness and the residue was dissolved in a definite volume of absolute alcohol to be used as sample for chromatographic process.

The Method in Comparison with Murakami's One—This chromatographical method was compared with Murakami's method by the recovery test in which pure free bile acids were added to human bile. As seen in Table IX, the present method and Murakami's one are equally reliable for the recovery test with free bile acid added over 4.0 mg. ; but below 4.0 mg. no cholic acid is detectable by Murakami's method and the value of desoxycholic acid obtained by it is considerably higher than the really value. Measured by the chromatographic method, on the contrary, both cholic and desoxycholic acid added in any amount larger than 0.2 mg. are accurately measurable.

TABLE IX

Comparison of This Method with Murakami's One

(Recovery test by the addition of free bile acids to human bile)

Bile H.K. (*Ulcus ventricule*)		Free bile acid added		Free bile acid determined	
Amount	Free bile acid content	Kind	Amount	Murakami's method	This method
ml.	*mg.*		*mg.*	*mg.*	*mg.*
0.5	0	Cholate	0.2	—	0.26
0.5	0	”	0.4	0	0.42
0.5	0	”	0.8	0	0.81
0.5	0	”	1.6	0	1.63
0.5	0	”	4.0	3.77	3.96
0.5	0	”	8.0	7.86	7.94
0.5	0	Desoxycholate	0.2	—	0.23
0.5	0	”	0.4	—	0.40
0.5	0	”	0.8	1.31	0.81
0.5	0	”	1.6	2.24	1.59
0.5	0	”	4.0	4.25	4.01
0.5	0	”	8.0	8.31	7.96

The two methods employed in the quantitative analysis of bile acid in natural bile gave the results given in Table X, approximately the same in one instance and somewhat at variance in another.

Results of the Determination of Bile Acid in Human Gallbladder Bile by This Method

The quantity of bile acids in gallbladder bile obtained at a time of laparotomy was differentially determined by this chromatographical method. The results are given in

Table X

Comparison of This Method with Murakami's One

(Results of the determination of bile acids in bile)

Cases	Method of determination	Conjugated bile acid determined (mg./ml.)		Free bile acid determined (mg./ml.)	
		Trihydroxycholanic	Dihydroxycholanic	Trihydroxycholanic	Dihydroxycholanic
U.D. *Cholelithiasis*	Murakami's	3.2	3.0	0.33	0.30
	This	3.3	3.1	0.27	0.22
G.T. *Cholelithiasis et Pancreatitis chron.*	Murakami's	11.2	21.6	0.66	0.27
	This	10.7	21.8	0.61	0.27

Table XI

Result of the Determination of Bile Acid in Human Gallbladder Bile

Case	Conjugated bile acid determined (*mg./ml.*)		Free bile acid determined (*ml./ml.*)	
	Trihydroxycholanic	Dihydroxycholanic	Trihydroxycholanic	Dihydroxycholanic
A.Y. *Ulcus ventriculi*	30.8	23.4	0	0
A.I. *Cholecystitis chron. sine* concremento	21.4	38.2	0	0
S.G. *Cholelithiasis*	9.0	3.7	0.33	0.13
M.N. ” ”	8.4	32.6	0.6	0.59
N.Y. ” ”	2.8	5.9	0	0.39
N.H. ” ”	2.8	4.0	0.35	0.22
K.Y. *Cholelithiasis et Pancreatitis chron.*	6.8	12.0	1.4	1.6

Table XI. The figures show that no free bile acids exists in normal bile and that it appears, though in a lower incidence than has hither-to been assumed, in cholelithiasis and pancreatitis ; the free bile acids consist of not only dihydroxycholanic acid but

also trihydroxycholanic acid, which has not been reported by any past researcher.

SUMMARY

In view of the important clinical significance of the appearance of free bile acid in bile, a method for differential micro-determination of bile acids by using chromatogram has been devised. The advantages of this new method are summarized as follows:

1. Any minute quantity of free bile acid contained in a small amount of human bile is quantitatively determinable.
2. By Murakami's colorimetric determination by using vanillin phosphoric acid reagents, the quantity of trihydroxycholanic acids and that of total dihydroxycholanic acids are determined separately, one as the free acid and the other as the conjugated acid. As dexosoycholic acid and chenodesoxycholic acid can not be separately determined, they are taken as a whole in determination.
3. The recovery of free bile acid added to human bile is *ca.* 99 per cent.

In conclusion, the author expresses his deep gratitude to Prof. H. Miyake and Prof. T. Shimizu, president of Okayama University, for their directions throughout this research, and also to Dr. E. Murakami, former lecturer of our Department, and Mr. M. Funatsu, assistant Prof. of Biochemistry of Kyushu University for their kind advices.

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BIOSYNTHESIS OF THREONINE FROM HOMOSERINE

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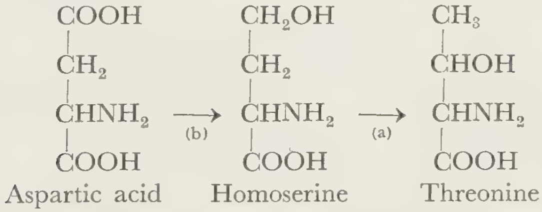
Homoserine has been considered as a precursor for both threonine and methionine since the pioneering investigation of Teas *et al.* (*1*, *2*). They observed that mutants of *Neurospora* and *Bacillus subtilis*, which require threonine for their growth, were able to grow if supplied with homoserine.

Recently, further studies concerning threonine synthesis were carried out by isotopic method ; Abelson (*3*, *4*), investigating metabolic relationship among a number of amino acids in *Escherichia coli* by means of the isotopic competition method with $C^{14}O_2$ or C^{14} glucose as the source of radioactivity, observed that added aspartic acid diminished the radioactivity of the protein aspartic acid and threonine to the same extent, and homoserine also had a pronounced effect on the radioactivity of threonine but none on aspartic acid, and suggested that homoserine is an intermediate between aspartic acid and threonine, as shown in the following scheme.



$$\begin{array}{ccccc} \mathrm{COOH} & & \mathrm{CH_2OH} & & \mathrm{CH_3} \\ | & & | & & | \\ \mathrm{CH_2} & & \mathrm{CH_2} & & \mathrm{CHOH} \\ | & & | & & | \\ \mathrm{CHNH_2} & \xrightarrow[\text{(b)}]{} & \mathrm{CHNH_2} & \xrightarrow[\text{(a)}]{} & \mathrm{CHNH_2} \\ | & & | & & | \\ \mathrm{COOH} & & \mathrm{COOH} & & \mathrm{COOH} \\ \text{Aspartic acid} & & \text{Homoserine} & & \text{Threonine} \end{array}$$

This result is consistent with other isotopic experiments involving the study with *Torula utilis* described by Ehrensvärd (*5*) and with *Escherichia coli* by Deluva (*6*) and McQuillen (*7*).

In our previous report (8) preliminary to this paper, we have demonstrated that the Reaction (a), a conversion of homoserine to threonine, is catalyzed by cell-free extract of yeast. It is of interest to note that the presence of adenosine triphosphate (ATP) and Mg^{++} were necessory for the activity of enzyme. During the course of our investigation, Cohen (*9*) showed that cell suspension of *Escherichia coli* catalyzed the Reactions (a) and (b) in the presence of glucose and phosphate under aerobic condition.

In the present paper, a preparing method of the enzyme from yeast and the details of its properties are described.

While this work was being prepared for publication, two papers were published by Cohen (*10, 11*) in which enzymatic threonine synthesis was described, using the extracts of *Escherichia coli.*

EXPERIMENTAL

Materials and Methods

Experiments with Intact Cells—A strain of *Saccharomyces cerevisiae* or *Torula utilis* was grown in a medium containing glucose, ammonium-sulfate, yeast extract and inorganic salts at 30° for 24 hours on a shaking machine. The yeast cells were harvested by centrifugation and washed twice with distilled water, and resuspended in 0.05 *M* phosphate buffer at pH 6.8 to permit convenient addition of 250 to 500 μg. yeast N to each of the reaction vessels.

DL-Homoserine and glucose were added to yeast suspension and incubated in a test-tube (20 × 160 mm.) aerobically with shaking at 37°. The details of the incubation system were shown in Tables I and II. At the end of incubation the reaction was stopped by heating the vessels in a boiling water bath for 5 minutes, and insoluble materials were removed by centrifugation. Two 0.5 ml. aliquots of the supernatant were taken for threonine assay. The blank correction was tested in the identical manner except that homoserine was omitted from the reaction mixture.

Experiments with Enzyme Solution—In early experiments (*8*) the enzyme has been extracted with phosphate buffer from fresh yeast ground with alumina. These extracts were sometimes found to contain considerable amounts of free threonine and some substances which interfered with the colorimetric determination of threonine. In the experiments reported here, the enzyme prepared from acetone powder of commercial baker's yeast was employed : 5.0 g. of acetone dried yeast, which was finely powdered in a mortar, was extracted with 50 ml. of cold 0.05 *M* K phosphate buffer (pH 7.0) in a cellophane bag for 48 hours at 0°. In the course of extraction, the bag was dialyzed against 1000 ml. of the same buffer, which was changed at least twice a day. After dialysis the content of the bag was centrifuged and the precipitate was discarded. A stable enzyme preparation with no loss in activity, was obtained by lyophylizing the dialyzed extract, and the dried material (stored in a desiccator at room temperature), did not show any diminution of activity at least for 2 months, while preparations stored at 0° to 5° in aqueous solution lost most activity in a week. Dialysis served not only to increase the specific activity of enzymes, but also removed substances interfering in the chemical determination of threonine.

The assay system used for the enzymatic activity, consisted of 10μM of DL-homoserine, 6 μM of ATP, 75 μM of K phosphate, 10 μM $MgCl_2$ and lyophilized enzyme in a final volume of 2.0 ml. The other additions and variations were noted in figures. After the reaction mixture was incubated at 37°, sample was deproteinized by heating at 90° for 5 minutes. The further procedure was the same as described in the case of intact cells. The threonine deaminase activity in this enzyme preparation was almost negligible at a neutral pH employed for the threonine synthesis.

Determination of Threonine—Threonine was determined at microgram levels by the procedure essentially as described by Winnick (*12*) and Neidig (*13*) with slight modifications : A sample to be analyzed was treated with neutral periodate solution in the outchamber of a Conway unit, and the acetaldehyde resulted from the oxidation of threonine was allowed to diffuse into bisulfite and was determined colorimetrically.

In some experiments, threonine formed was also determined by biological assay with *Leuconostoc mesenteroides* which did not show any response to such C_4-compounds, as homoserine, D-threonine, DL-allothreonine, α-ketobutyric acid, α-keto-β-hydroxybutyric acid and α-keto-β-hydroxybutyric acid amide in a threonine deficient medium.

Chemical Preparations—DL-Homoserine was synthesized by the method of Armstrong (*14*). D- and L-isomers of homoserine were prepared by the procedure described by Weiss and Stekol (*15*), they were optically pure more than 99.5 per cent. ATP was obtained from Cell Stoff Fabrik Waldhof or isolated as the barium salt from rabbit muscle. The solution of sodium salt of ATP made from either source of the barium salts was tested by the Beckman spectrophotometer at 260μm., and was found to have correct amount of 10 minutes phosphate.

RESULTS

Experiments with Intact Cells—Table I shows L-threonine synthesis from homoserine

TABLE I

Threonine Synthesis from Homoserine by Yeast Cell Suspension

Time	Threonine synthesized		
	Torula utilis	*Sacch. cerevisiae*	
min.	μM	μM	μM
30	0.23*	0.30*	0.31†
60	0.50	0.43	0.42
90	—	0.59	0.58
120	1.05	0.71	0.69
150	1.12	—	—

Values expressed in μM of threonine synthesized per 0.25 mg. of yeast-N. Incubation mixture (2.0 ml.) contained 5 μM of DL-homoserine, 15 μM of glucose, 0.25 to 0.30 mg.-N of yeast, 25 μM of K phosphate (pH 6.8). Incubated at 37° under aeration.

* chemically determined, † bioassayed.

catalyzed by cell suspension of *Torula utilis* and *Saccharomyces cerevisiae* under the condition favouring maximal synthesis. The rate of reaction was stimulated remarkably by the addition of glucose under an aerobic condition. If the air of gas phase was substituted for by nitrogen, activity was decreased to less than a half of that obtained in air, even in the presence of glucose.

In the experiments with *Saccharomyces cerevisiae* the samples taken at each period of incubation were tested by both chemical and biological methods. As can be seen in Table I, there is good agreement between two series of values obtained different assay methods.

The dependence of the rate of threonine synthesis on the concentration of yeast nitrogen is shown in Table II. Under the conditions of the test, the rate of threonine synthesis is proportional to the cell concentration at low levels of it. The assay of the activity of cell suspension was usually carried out at concentrations corresponding to the initial rather linear part of the curve. The yeast cells were active over a broad pH range, with an optimum at pH 6.5 to 7.0 (Fig. 1).

TABLE II

Effect of Yeast Concentration

Suspension of *T. utilis*	Threonine synthesized
µg.-N	µM
27.5	0.16
55	0.26
110	0.40
275	0.68
550	0.89
1110	0.90

In this experiment each vessel contained 15 µM of DL-homoserine. Incubated at 39° for 120 minutes. Other conditions as in Table I.

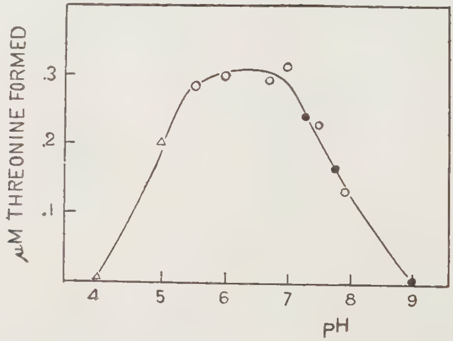


FIG. 1. Effect of pH on threonine synthesis by yeast cell suspension.

Incubated at 37° for 90 minutes, other conditions as in Table I.

Threonine deaminase activity of yeast cell suspension was found to be negligible under the condition employed in this study.

Experiments with Enzyme Preparation

Activation of Threonine Synthesis by ATP—It will be possible to mention that the conversion of L-homoserine to L-threonine is coupled with energy yielding system, as might be expected from experiments with intact cells. In fact, ATP was found to be an essential component of a threonine synthesizing system when cell-free enzyme was employed. If ATP was omitted from the reaction mixture, no synthesis of threonine was observed. Addition of glucose and aeration of the system were no more effective to the soluble enzyme system in the presence of ATP. Fig. 2 shows the effect of ATP concentration on the rate of the reaction.

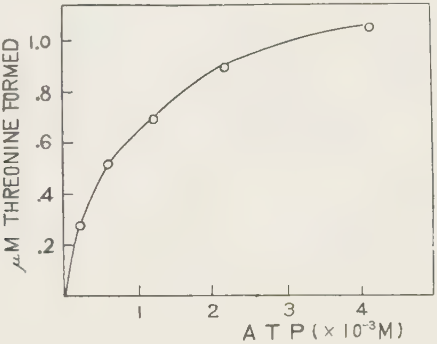


FIG. 2. Effect of varying ATP concentration.

The assay conducted under standard conditions as described in the text except for the variation of ATP. Incubated for 120 min. Each vessel contained 1.2 mg. of enzyme-N.

Effect of Enzyme Concentration—The effect of enzyme concentration was investigated at six different concentrations of enzyme ranging from 75 to 1200 μg. N in the presence of excessive amount of substrate. The result is shown in Fig. 3. The rate of threonine synthesis was roughly proportional to the enzyme concentration except higher concentration than those usually employed in our experiments.

Optical Specificity and Effect of Homoserine Concentration—The relationship between the amount of homoserine and rate of threonine formation was investigated at six different concentrations of DL-homoserine (Fig. 4), and at three different concentrations of D- and L-homoserine (Table III). In the latter experiments threonine formed was determined by two different assay methods. The values obtained by biological assay represent only the amount of L-threonine synthesized among four isomers which involve D- or L-threonine and D- or L-allothreonine. On the other hand, chemical determination makes no distinction between four isomers. In our experiments, as is shown in Table III, two series of values obtained by different assay methods were in good agreement. Accordingly, it may be possible to conclude that the diastereoisomeric configuration of the threonine synthesized is restricted to only that of L-threonine. D-Homoserine served as neither substrate nor inhibitor of the reaction. Thus it may be decided that only L-isomer of homoserine is converted to L-threonine without the formation of any other three isomers of threonine.

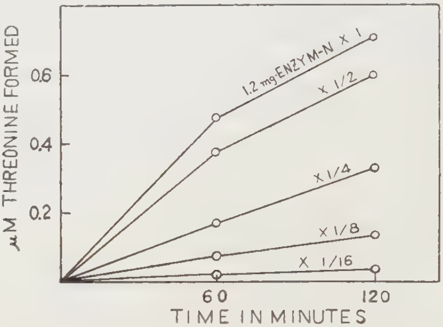


FIG. 3. Effect of time and varying enzyme concentration.
Each vessel contained DL-homoserine 15 μM, $MgCl_2$ 10 μM, other conditions were the same as in the text.

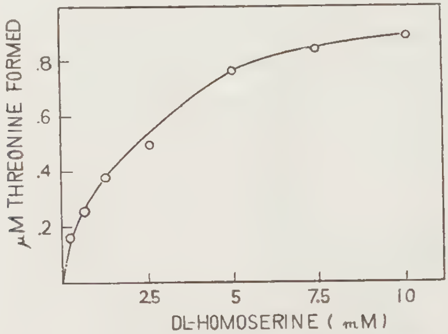


FIG. 4. Effect of varying homoserine concentration.
Each vessel contained 0.77 mg. of enzyme-N. Incubated for 120 min. Other conditions as in the text.

Activation by Mg^{++} and Mn^{++}—The activation by Mg^{++} is illustrated in Fig. 5. Without added Mg^{++} the activity of the enzyme is almost negligible when the well dialyzed

TABLE III

Optical Specificity of the Enzyme

Substrate	Amount of substrate added	Threonine formed	
		Chemical assay	Bioassay
	μM	μM	μM
L-Homoserine	0.63 2.50 10.00	0.31 0.62 0.75	0.30 0.63 0.79
D-Homoserine	0.63 2.50 10.00	0 0 0	0 0 0
DL-Homoserine	1.25 5.00 20.00	0.29 0.63 0.75	0.30 0.67 0.76

Values are expressed in μM threonine synthesized per vessel. Each vessel contained 0.65 mg. of enzyme-N. Incubated for 120 minutes, other conditions were the same as in the text except for the variation of homoserine.

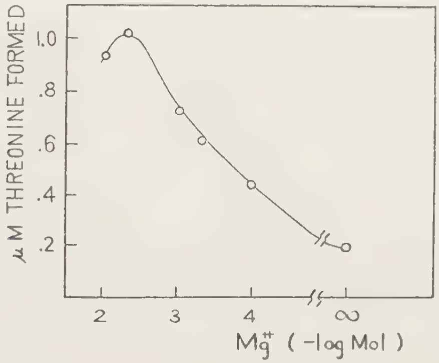


FIG. 5. Effect of varying Mg++ concentration.

Enzyme solution was dialyzed against phosphate buffer for 76 hours before use. Each vessel contained 0.8 mg. of enzyme-N. Incubated for 120 min. Other conditions as in the text.

enzyme is employed. Under the conditions tested the optimal Mg^{++} concentration was found to be around $5\times10^{-3}M$, higher concentrations were somewhat inhibitory.

Mg^{++} may be replaced by Mn^{++} at a similar level of concentration. However, the values which correspond to valid concentrations of Mn^{++} were not reliable, because of the formation of insoluble manganese-phosphate compound under the conditions employed in our experiments.

Effect of pH—The maximal rate of threonine formation occured at pH 6.5 to pH 7.0 (Fig. 6).

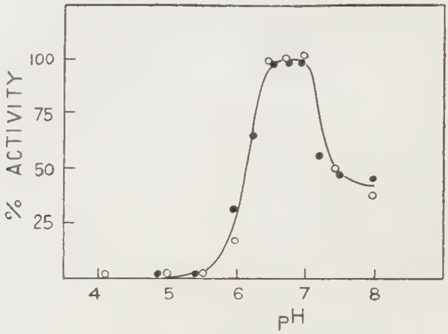


FIG. 6. Effect of pH.

Composite results of two experimental series with different enzyme preparations. The assay conducted under standard conditions. Incubated for 120 min.

Inhibitors—The enzyme was completely inhibited by *p*-chloromercuribenzoate at a concentration of $10^{-3}M$. Monoiodoacetamide also decreases the rate of reaction about 50 per cent at a concentration of $10^{-2}M$. These observations may suggest the role of sulfhydryl group of enzyme protein for its activity. On the other hand, however, glutathione and HCN, which are generally known to activate sulfhydryl enzymes, inhibited the reaction strongly. This curious observation might lead to the consideration that there are more than one enzyme involved in this reaction. But it does not seem worthwhile to discuss these results until the series can be extended by using a more purified enzyme preparation. Also, 2,4-dinitrophenol inhibited considerably at a concentration of $10^{-3}M$, but NaF, NaN_3 and malonate had little or no effect on the activity. Inhibition of arsenite was somewhat equivocal (Table IV and Fig. 7).

Occurrence of the Enzyme—Testing with homoserine as a substrate, the enzyme was found in some microorganism, but not in liver and kidney of a rat (Table V).

Since these observations were made at a time when optimal test conditions had not been worked out, reliable values are not available. However, on the basis of comparison with yeast cell suspension tested under comparable conditions, some of these cell suspensions were 1/10 to 1/2 as active.

DISCUSSION

Though it is not apparent whether the conversion of L-homoserine to L-threonine is catalyzed by one enzyme or enzyme system, the finding

TABLE IV

Effects of Inhibitors on Threonine Synthesis

Compound	Concentration	Inhibition*
	M	%
2,4-Dinitrophenol	1×10^{-3}	46
Sodium azide	1×10^{-2}	3
Sodium fluoride	1×10^{-2}	0
Sodium arsenite	1×10^{-2}	20
Hydrogen cyanide	1×10^{-2}	73
Glutathione	1×10^{-2}	67
Cysteine	1×10^{-2}	65
p-Chloromercuribenzoic acid	1×10^{-3}	100
Monoiodoacetamide	1×10^{-2}	50
Sodium malonate	1×10^{-2}	0

* Mean value of two or three experimental series.

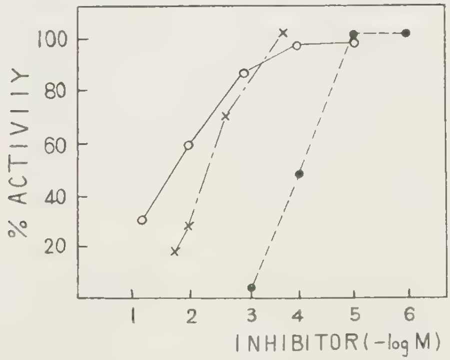


FIG. 7. Effect of varying concentration of inhibitors.

Solutions of inhibitors were adjusted to pH 7.0 before use. Each vessel contained 0.78 mg. of enzyme-N., incubated for 120 min. Other conditions as in the text. ○ Monoiodoacetamide, × Glutathione, ● *p*-Chloromercuribenzoate.

that ATP is required favors the idea that the phosphorylation of intermediate may take place in the course of the reaction. The exact point in the synthesis at which ATP functions is yet to be clarified. Further detailed experiments are now in progress.

The enzyme (or enzyme system) appears to have rather broad distribution in microorganisms, such as *Aspergillus oryzae*, *A. niger*, *A. glaucus*, *A. ochraceus*, *A. flavus*, *Penicillum purpurogenum* and especially in yeast as are shown in Table V, *Neurospora* (*1*), *Bacillus subtilis* (*2*) and

TABLE V

Occurrence of Threonine Synthesis from Homoserine in Microorganisms

Organism	Test condition	Threonine formed
		μg.
Asp. oryzae 03-6	Cell suspension	8
Asp. oryzae 08-1	”	7
Asp. niger 1-6	”	4
Asp. glaucus	”	4
Asp. ochraceus	”	0
Asp. flavus A7-3	”	4
Pen. purpurogenum 722	”	38
Pen. roqueforti 731	”	0
Rat liver	Slice†	0
	Homogenate*	0
Rat kidney	Slice†	0
	Homogenate*	0
Rat spleen	Slice†	0

Mycelial fungi were grown on a synthetic medium with shaking for 24 hours, being collected by centrifugation, cells were incubated aerobically in a medium containing 5 μM of DL-homoserine, 50 μM of K phosphate and 28 μM of glucose in a total volume of 2.0 ml., at 37° for 60 minutes. Values are expressed μg. of threonine formed per 0.25 mg. of mycelial nitrogen in an hour.

† 50 mg. to 100 mg. of slices of fresh rat organ were incubated aerobically in a medium which contains 40 μM of DL-homoserine, 50 μM of K phosphate, 10 μM of glucose and 13.5 mg. of NaCl in a total volume of 2.0 ml. at 37° for 30 to 60 min.

* Rat organ was homogenized in a chilled Potter-Elvehjem glass homogenizer with the same volume of 0.05 M phosphate buffer (pH 6.8). A assay system consisted of 5 μM of DL-homoserine, 50 μM of K phosphate, 6 μM of ATP 10 μM of $MgCl_2$ and 0.5 ml. of homogenate in a total volume of 2.0 ml. was incubated at 37° for 60 to 120 minutes.

Escherichia coli (*9, 10,11*). These results may indicate that the conversion of homoserine to threonine is an important pathway of threonine synthesis in microorganisms. On the other hand, this reaction seems to occur neither in homogenate nor slice of liver and kidney of a rat, even in the presence of either ATP or glucose. The failure of the threonine synthesis in a rat under the condition under which many sorts of microorganism are able to do this, may explain why threonine is an essential amino acid for animal. Previously, McCoy *et al.* showed that homoserine was not able to replace threonine for the growth of young rats, when added in the threonine dificient diet (*16*). Binkley *et al*, (*17*) and Carroll *et al.* (*18*) reported the presence of homoserine deaminase in a rat liver, which deaminate homoserine into α-ketobutyrate and ammonia. These findings, together with our observations above described, may suggest that threonine cannot be synthesized from homoserine in a rat, because the enzyme involved in this transformation is absent and homoserine, if it was given or synthesized, would be deaminated into α-ketobutyrate.

SUMMARY

The conversion of L-homoserine to L-threonine was catalyzed by yeast cell suspension in the presence of glucose under aeration. The stable enzyme preparation was prepared from acetone powder of yeast. The properties and requirements of this enzyme system are described.

1. ATP is an indispensable component for the enzyme activity.
2. Mg^{++} stimulates the reaction remarkably. The optimal concentration is about $5 \times 10^{-3} M$. Mg^{++} may be replaced by Mn^{++}.
3. Optimal pH of this reaction ranges at pH 6.5 to 7.0.
4. Only L-isomer of homoserine is utilized for threonine synthesis. D-Homoserine is not effective. The threonine synthesized by this enzyme is found to be only L-threonine, other isomers of threonine are not formed.
5. The reaction is completely inhibited by *p*-chloromercuribenzoic acid at a concentration of $1 \times 10^{-3} M$.
6. The enzyme appears to have wide distribution in microorganisms but is not present in a rat.

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The Journal of Biochemistry, Vol. 42, No. 2, 1955

ON THE INTERACTION OF CYCLIC AMINO ACIDS WITH ASCORBIC ACID. I

By KENZO TORII and TADASHIGE MORIYAMA

(From the Department of Biochemistry, Nara Medical University, Nara)

(Received for publication, January 7, 1955)

In this report we will deal chiefly with the data obtained during the study on tryptophan metabolism, and also partially the data about tyrosine and histidine metabolism.

It was found that in some cases the values of tryptophan observed in the solution were always about 30 per cent lower than those expected theoretically. Examining where it started from, it was conclusively ascribed to co-existing ascorbic acid. Results are shown in Fig. 1, whereby tryptophan was determined by Spies' method (*1*) (here refered to as DAB reaction).

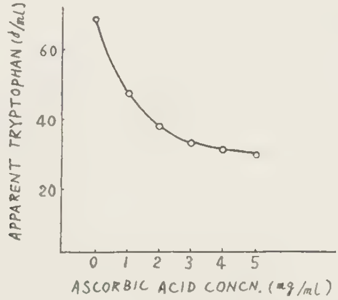

Fig. 1. Effect of A.A. addition on tryptophane values

Reasonable interpretation may be one of the following: a) *p*-Dimethylaminobenzaldehyde (DAB), combining with ascorbic acid, inhibits color developments ; b) decrease of the obtained values is due to the interaction between tryptophan and ascorbic acid ; c) something else inhibits the color-developing mechanism.

In order to invest which is the case, we ran the following experiments.

EXPERIMENTAL

Experiment I.

By use of a Beckman type spectrophotometer (Hitachi Co., Ltd.), the absorption curves were obtained with the solutions containing the materials of items (1)∼(4), respectively. (*cf.* Figs. 2 and 3)

(1) Tryptophan+DAB
(2) Tryptophan+ascorbic acid+DAB,
(3) Ascorbic acid+DAB,
(4) H_2O+DAB,

As demonstrated, the results with the mixtures of (3) and (4) fall nearly on the same curve and those of (1) and (2) differ considerably. These facts suggest that no combination takes place between ascorbic acid and DAB, but there exists some interaction between tryptophan and ascorbic acid.

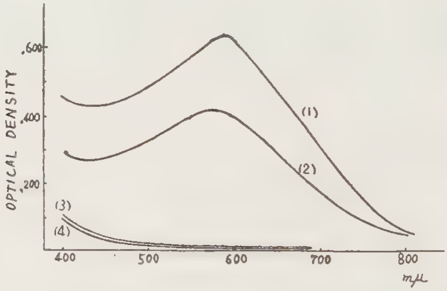

FIG. 2. Absorption spectra curves (control: H_2O)

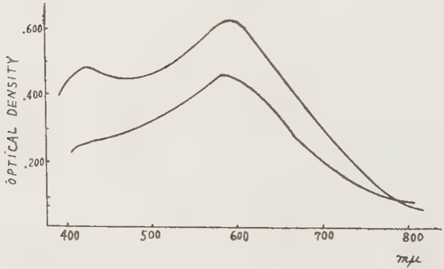

FIG. 3. Absorption spectra curves (control: DAB sol.)

Room temp. 15°, 80% phenol, ninhydrine reaction

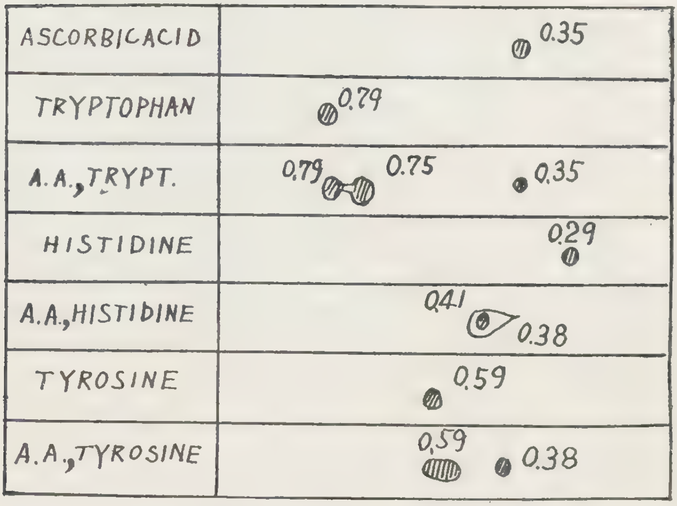

Fig. 4. Paper-chromatograms.

Experiment II

In addition, paper-chromatographic experiments (*2*), were carried out by using Toyo filter paper, No. 50, and phenol as a developing agent; ninhydrin reaction was chiefly employed for color development, and Ehrlich's and Pauli's reactions were also used, both being shown in parallelism.

The rates of flow (Rfs.) of 0.35 (brown red), 0.75 (brown red) and 0.79 (pale violet) were observed for the mixture of tryptophan and ascorbic acid. Tryptophan alone shows a spot at Rf=0.79. Though the difference between two Rfs., *i.e.* 0.79 and 0.75, is so small, it could be considered that a substance other than tryptophan might have been formed, because the color tone differs from each other.

This supports our view of the combination of the two.

Experiment III

DAB reaction was applied to abrine (*N*-methyltryptophan) and there were obtained lower values similar to tryptophane (Fig. 5). Paper-chromatographic technique showed clearly a new brown red spot of Rf=0.75.

Experiment IV

Experiment III suggests that the reaction took place not in the side chain, but in

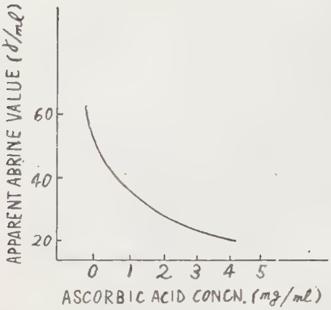

FIG. 5. Effect of A.A. addition on abrine values

the nucleus. Figs. 6 and 7 show the results in the cases of tryosine and histidine, respectively. Tyrosine was determined by Lugg's method (3), and histidine by the modified Pauli's method (4).

As shown in the figures, it tends to "saturation" as ascorbic acid increases. The approximate estimation of molecular ratio when saturation has been established, is shown in Table I.

From the above mentioned, it can be conclusion that the role of ascorbic acid toward cyclic amino acid should consist in its loose combining with these 3 amino acids, masking the color developing reaction and then splitting their nuclei oxidatively.

TABLE I

Histidine : A.A.	1 : 2
Tyrosine : A.A.	1 : 10
Tryptophan : A.A.	1 : 32

The approximate estimation of molecular ratio when saturation has been established.

FIG. 6

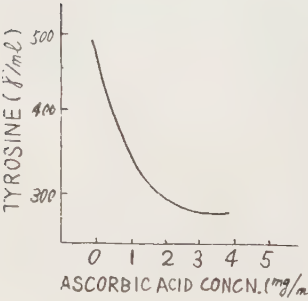

FIG. 6. Effect of A. A. addition on tyrosine values

FIG. 7

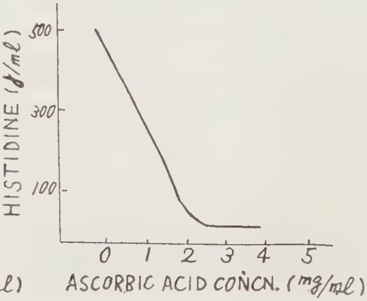

FIG. 7. Effect of A. A. addition on histidine values

Experiment V

The mixtures of each 5 mg. of tryptophan with 5, 10, 20 and 100 mg. of ascorbic acid, were separately placed in Folin tubes at 37° in a thermostat with air-bubbling through them and the changes in the amounts of tryptophan were plotted against time. The formation of kynureine-like substance was confirmed.

Kynurenine was determined by Itou-Mochizuki's reaction (*5*) in which Tsuda's reagent coupled with kynurenine (Table II).

Table II

Relation with A.A. Concentration

A.A. *mg.* / Time *hrs.*	1	5	10	20	50	100
0	0	0	0	0	0	0
1	0	2.4	1.5	0.5	0	0
2	0	4.0	3.5	0.7	0	0
3	0	4.3	4.2	1.0	0	0
4	0	4.5	5.2	2.7	0	0

The numeral values in the table: kynurenine formation in γ/ml.
Tryptophan concentration‡ 0.2 mg./ml.

In the cases of the addition of 50 and 100 mg. of ascorbic acid to 5 mg. of tryptophan, kynurenine-like substance was not detectable but a considerable decrease of trypophane was observed. This may be due to the fact that kynurenine-like substance might have further suffered subsequent splitting of the nucleus.

The absorption curve of the kynurenine-like substance is shown in Fig. 8. It was likely to correspond roughly to that of kynurenine, though any crystalline substance has not yet been obtained.

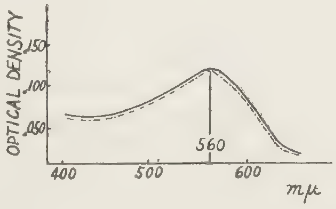

Fig. 8. Absorption curves
Full line: kynurenine Broken line: Kynurenine-like substance
The absorption curve of the kynurenine-like substance corresponds roughly to that of kynurenine.

DISCUSSION

Ascorbic acid is one of the vitamins which are required in a larger amount. Amino acids are stable *in vitro*, whereas they are unstable and readily decomposed *in vivo*. Edelbacher reported that histidine was decomposed on mixing with ascorbic acid (*11*). Imanaga confirmed that this decomposed product was aspartic acid, using paper chromatographic technique (*10*). Sealock described that the role of ascorbic acid towards tyrosine splitting consisted in introducing oxygen successively by means of endiol formation (*6*).

By his experiment Takagi showed that ascorbic acid could take the place of anthranilic acid partially (*7*).

This fact may be explained by assuming that ascorbic acid which is necessiated in the course of transformation of tryptophan to anthranilic acids, *i.e.* for the opening of pyrrol nucleus, is not required and spared.

On the other hand tryptophane has been transformed into kynurenine *in vitro* by Otani through $KMnO_4$ oxidation (*8*) and by Amano through ozone oxidation (*9*). And Imanaga confirmed H_2O_2 formation from ascorbic acid. Consequently, as shown in Otani and Amano's experiments, it is most probable that transformation of tryptophan can occur oxidatively in the presence of ascorbic acid. So it seems to us that there is still some room for discussing once again the view that hydrogen peroxide is very toxic and not available for living body and that, once formed, it is instantly destroyed by peroxidase or catalase.

SUMMARY

1. Coexistence of ascorbic acid in the solution of tryptophan, histidine or tyrosine caused apparent lowering of the observed values of amino acids. Workers in these field, should make some allowance for in the experiments of enzymological decomposition of amino acids by using the homogenate of various organs.

2. Kynurenine could be detected, by air-bubbling through the mixture of tryptophan and ascorbic acid.

3. A new spot was confirmed on the paper-chromatogram of the mixture of tryptophan and ascorbic acid.

It is planned to study further the quantitative relation and the mechanism of nucleic splitting.

Particular thanks are due to Prof. K. Ichihara in Osaka University who has kindly given us abrine. The authors wish to thank Prof. N. Ito for his guidance.

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ON THE INTERACTION OF CYCLIC AMINO ACIDS WITH ASCORBIC ACID. II

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(Received for publication, January 7, 1955)

It has been reported that the mixed solution of tryptophan and ascorbic acid shows apparently decreased values of tryptophan by use of Spie's *p*-dimethylaminobenzaldehyde (DAB) reaction, together with the appearance of a new spot on the paper-chromatogram (*1*).

These reactions are interpreted as follows: tryptophane might not simply interrupt a mechanism of color-development, but it might be combined with ascorbic acid, masking the colorization. In this paper, we intend to report the influences of pH, Cu^{++} and Fe^{+++} ions upon kynurenine production in the case of air-bubbling through the tryptophan-ascorbic acid mixture.

If ascorbic acid would at first form hydrogen peroxide, followed by the oxidation of tryptophan to kynurnine, an additional use of colloidal platinum for destruction of hydrogen peroxide would perfectly inhibit the production of kynurenine. The following experiments were carried out in order to know whether the above assumption may have been true or not. The method for the determination of tryptophan and kynurenine has been already described (*1*).

EXPERIMENTALS

Experiment I

In the previous reports the experiments were performed by using common distilled water, and this time the redistilled water was employed, for fear that a trace of metal ions should give some effects.

All the results agreed, however, quite well with the experiments reported previously.

The data are shown on Table I. The spectral maxima and absorption curves were quite identical with those of kynurenine solutions (Fig. 1), and the pH values in the final mixture (after 6 hours) was about 4.0.

TABLE I–a

Tryptophan Values when Air-bubbling Was Carried Out

Group / Time *hrs.*		0	2	4	6	22
Tryptophan + A.A.	Optical density	0.120	0.215	0.580	0.870	0.930
	Tryptophan concn. *r/ml.*	18.0	32.2	87.0	130.5	138.5
Tryptophan alone	Optical density	1.51	1.53	1.55	1.55	1.55
	Tryptophan concn. *r/ml.*	226	229	230	230	230

Tryptophan concn. : 0.25 mg./ml.
A.A. concn. : 0.4 mg./ml.

TABLF I–b

Kynurenine Values when Air-bubbling Was Carried Out

Group / Time *hrs.*		0	2	2	4	6
Tryptophan + A.A.	Optical density	0.011	0.034	0.045	0.051	0.067
	Tryptophan concn. *r/ml.*	0.7	0.25	3.5	4.0	5.1
Tryptophan alone	Optical density	0.000	0.000	0.000	0.000	0.000
	Tryptophan concn. *r/ml.*	0	0	0	0	0

Solutions used here are the same as in Table I–a.

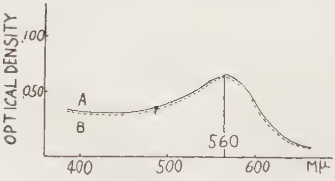

FIG. 1. Absorption curve
A : kynurenine solution
B : Material

Experiment II

Since the solution becomes acidic during air-bubbling through it, $M/15$ phosphate buffer solutions were used to keep the solution at pH 5.2, 6.5 and 8.4, respectively.

TABLE II–a

List of Conditions

Group No.	$M/15$ phosphate buffer	pH	Tryptophan	A.A.	Addition
1	25 *ml.*	5.2	9.09 *mg.*	3.6 *mg.*	—
2	25	6.5	9.09	3.6	—
3	25	8.5	9.09	3.6	
4	25	5.2	9.09	3.6	$CuSO_4$ 0.004 g.
5	25	5.2	9.09	3.6	$FeCl_2$ 0.004 g.
6	25	5.2	9.09		$CuSO_4$ 0.002 g.

The results are shown in Table II–a. The most convenient pH for kynurenine formation was found to be at 5.2.

When Cu^{++} ions existed in the solution, much kynurenine was produced even in a short time (about 3 times more than in Cu^{++}-free solution under the same condition), and the rate of reaction increased markedly. As shown in Table II, even in the tryptophane-Cu^{++} solution, kynurenine was not produced in the absence of ascorbic acid. Contrary to Cu^{++}, Fe^{+++} addition did not influence the reaction (Table II–b). Fig. 2 shows the absorption curves whereby the solution was air-bubbled for 6 hours under the condition of Table II–b, Nos. 1, 4 and 5, and then developed color.

TABLE II–b

Influence of pH, Cu^{++} and Fe^{+++}

(Kynurenine values) γ/cc.

Group No. / Time *min.*	1	2	3	4	5	6
0	5.8	3.2	1.4	16.4	6.4	0
30	4.9	3.2	1.9	21.4	6.0	0
60	5.5	4.0	1.6	22.2	6.4	0
180	6.6	3.6	2.0	22.2	7.0	0
360	8.1	4.8	2.2	22.9	7.0	0

The numeral values in the table : kynurenine formation in γ/ml.

Experiment III

The effect of the increasing amounts of either Cu^{++} or ascorbic acid on the reaction rate of kynurenine formation was studied. The results are shown in Table III.

Here, it was found that the production of kynurenine was dependent upon the in-

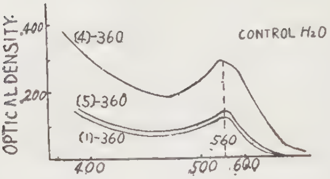

FIG. 2. Absorption curves of group 1, 4 and 5 after reaction. (360 min.)

The absorp. curves of these agree to kynurenine.

Kynurenine λ max.: 560 mμ.

creasing ascorbic acid concentration, but not upon that of Cu^{++} ions, reacting presumably as catalyst. At the end of bubbling, pH of the solution became more acidic than in the Cu^{++}-free solution.

TABLE III

Effect of Cu^{++} and A.A.

Group \ Time *min.*	0	30	60	180	360
Tryptophan+A.A. 1mg.	0	0	1.4	8.8	9.2
Tryptophan+A.A.+ $CuSO_4$ 1 mg.	0	2.0	2.8	28.0	28.0
Tryptophan+A.A. 60 mg.	1.1	24.4	27.8	33.4	33.5

Tryptophan concn.: 1 mg./ml. Numerical values in the table: kynurenine formation in γ/ml.

In the production of kynurenine, an increase in A.A. is more effective than that in Cu^{++} ions.

Experiment IV

If it is assumed that in the decomposing process of ascorbic acid, hydrogen peroxide may be formed, and then act on tryptophane, the addition of colloidal platinum should break down peroxide (*2*), so that kynurenine should not be produced.

Table IV shows such an experiment. There are clearly shown the decrease of kynurenine formation, amounting 70 per cent, and yet a little kynurenine is formed. When sodium cyanide was added under the anticipation of inhibition of colloidal platinum action, any cyanide inhibition was not detectable. In order to prevent the mixtures from alkalinizing due to addition of sodium cyanide, the mixture was buffered to pH 4.0 by adding KH_2PO_4.

TABLE IV

Influence of Colloidal Pt and KCN

Group / Time *min.*	0	60	180	360	end pH
Tryptophan+A.A.	1.2	1.2	1.6	8.5	4.0
Tryptophan+A.A.+ colloidal Pt	0.8	1.1	0.8	3.1	4.0
Tryptophan+A.A.+ colloidal Pt+KCN	0.7	0.8	1.1	2.2	4.0

Numeral values in the table : kynurenine formation in γ/ml.
By adding colloidal Pt, kynurenine formation was decreased.

DISCUSSION

Though the Cu^{++} content in the redistilled water was not quantitatively determined, it is conceivable that Cu^{++} ions acted as catalyst in the conversion of tryptophan to kynurenine in presence of ascorbic acid. In the case of Fe^{+++} ions, there is some probability that there was formed sparingly soluble ferric phosphate, diminishing the Fe^{+++} effect. In this point we are planning further studies using other buffers.

The complete inhibition of kynurenine formation by increasing colloidal platinum, was attempted in vain. The effect of KCN, an inhibitor of platinum catalysis, was also not detectable.

From the above experiments, it is convinced that tryptophan combines loosélly with ascorbic acid in the primary step and next, on the way to its decomposition to kynurenine, hydrogen peroxide is formed and secondarily acts upon the tryptophan-ascorbic acid-complex.

Knox and other (*3*) describes that they could not decompose tryptophane with commercial hydrogen peroxide. The authors assume that ascorbic acid may combine with tryptophan in 2 and 3 positions to form endiol.

SUMMARY

1. Within the range of our experiments, the optimum pH was at

kynurenine formation by air-bubbling through the mixture of tryptophan and ascorbid acid.

2. Cu^{++} ion was one of the catalyst in this reaction.

3. Inhibition of colloidal platinum in this reaction was about 70 per cent.

4. Action of KCN on colloidal platinum could not be recognized.

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UNTERSUCHUNG DER KRÖTENGALLE

VIII. ÜBER DIE REDUKTIONSPRODUKTE VON VERSCHIEDENEN GALLENSÄUREN UND STEROGALLENSÄUREN MITTELST NATRIUMÄTHYLAT

Von KENZI MATUMOTO

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(Der schriftleitung zugegangen am 25, Januar 1955)

Durch die verschiedenen Experimente wurde wahrscheinlich gemacht, dass die Norhomogallensäure (C_{24}) aus den Sterinen über einige Zwischenprodukte gebildet wird, was dadurch unterstützt wird, dass als intermediäres Stoffwechselprodukt des Sterins verschiedene Steroide und Sterogallensäuren in der Galle vorkommen und dass diese Steroide und Sterogallensäure chemisch konstitutionell mit der Norhomogallensäure in inniger Beziehung stehen und sogar einige davon künstlich in Cholsäure abgebaut werden können.

Einerseits um dies sicher festzulegen und andrerseits um die Kohlenstoffzahl dieser Zwischenprodukte festzustellen, wurden die intermediären Stoffwechselprodukte des Sterins durch Reduktion ihres Säureesters mit Natriumaethylat in Alkohol verwandelt und im Verglich mit den bekannten Alkoholen identifiziert.

Aus der Krötengalle wurden Trioxy-bufosterocholensäure $C_{28}H_{46}O_5$ (Shimizu u. Oda (1934) (*1*) und Trioxy-isosterocholensäure $C_{28}O_{46}$ O_5 (Shimizu u. Kazuno (1936) (*2*) isoliert, deren chemische Konstitution bzw. die der Seitenketten noch nicht feststeht. Aus derselben Galle wurde auch Tetraoxy-norbufostan $C_{27}H_{48}O_4$ von Makino (1933) (*3*) gefunden, dessen Kohlenstoffzahl nach ihm 27 betragen soll.

Durch Vergleich des Tetraoxy-norbufostans mit dem durch Reduktion des Trioxy-bufosterocholansäureesters oder Trioxy-isosterocholansäureesters gewonnenen Alkohol konnte der Unterschied oder die Identität der beiden Alkohole erkannt werden.

Trioxy-bufosterocholensäure-aethylester vom Schmelzpt. 153° wurde mittelst Natriumaethylats in Tetraoxy-bufosten (Fp. 216°) reduziert und daraus weiter durch katalytische Hydrierung mit Wasserstoff Tetraoxy-bufostan vom Schmelzpt. 216° gewonnen. Ebengalls wurde aus Trioxy-isosterocholensäureaethylester (173°) über Tetraoxy-isobufosten (220°)

Tetraoxy-isobufostan vom Schmelzpt. 215° erhalten. Tetraoxy-isobufostan schmilzt bei der Mischprobe mit Tetraoxy-bufostan bei 208°. Die beiden Alkohole sind also voneinander ganz verschieden und der Unterschied zwischen beiden beruht höchstwahrscheinlich auf der Verschiedenheit der Struktur von Seitenketten wie bereits in voriger Mitteilung erwähnt wurde (Schimizu u. Kazuno (1937) (*4*).

Die beiden Steroide zeigen auch bei der Mischprobe mit Tetraoxy-norbufostan von Fp. 230° eine Schmelzpunktdepression. Die beiden Tetraoxy-bufostan und Tetraoxy-norbufostan wurden durch Chromsäure in Ketocarbonsäure oxydiert und diese Ketocarbonsäuren miteinander verglichen. Weiter wurden einige Derivate der Sterocholensäure hergestellt und hier auch miteinander verglichen.

Der Übersichtlichkeit halber wurden diese verschiedenen Derivate der Sterocholensäure und der Steroide in folgender Tabelle I zusammengestellt.

In genau gleicher Weise wurden die verschiedenen Alkohole aus dem Aethylester von Cholsäure, Desoxycholsäure, Hyodesoxycholsäure, Ursodexoxycholsäure, Chenodesoxycholsäure und Lithocholsäure synthetisiert und in folgender Tabelle II zusammengestellt.

Das aus Desoxycholsäure dargestellte 3,12-Dioxycholanol-24 wurde aus Alkohol-Wasser oder aus Eisessig-Wasser umkrystallisiert. Dieser Krystall sintert bei 95° und schmilzt bei 105°. Dies beruht auf dem Krystallwasser und zwar hat der Alkohol 2 Mole Krystallwasser. Der Alkohol bildet also keine Choleinsäure. Für die Choleinsäurebildung der Desoxycholsäure ist also das Vorhandensein der Carboxylgruppe und der α-ständigen HO-Gruppe in der Stellung an C_3 des Moleküls notwendig.

Zur Nomenklatur (*Shimizu, Kazuno u. Matumoto*)

Bei der Gelegenheit der Darstellung des Sterocholans $C_{28}H_{50}$ möchten wir eine einfache und allgemeine Nomenklatur vorschlagen. Den Kohlenwasserstoff $C_{28}H_{50}$ bezeichnen wir als Sterocholan, weiter den $C_{27}H_{48}$ als Nor- den $C_{26}H_{46}$ als Bisnor- (Bufostan, Isobufostan, Norbufostan, und Bisnorbufostan) und den $C_{25}H_{44}$ als Homocholan und Iso-homocholan (Okasaki 1934) (*5*), die gesättigte Stammsäure $C_{28}H_{48}O_2$ als Sterocholansäure gegenüber der Cholansäure $C_{24}H_{40}O_2$, die Trioxy-bufosterocholensäure selbst demgemäss einfach als 3,7,12-Trioxy-sterocholensäure. Die Bezeichnung neuer Derivate ergibt sich von selbst, wie das $C_{24}H_{42}$ als Cholan, das $C_{23}H_{40}$ als Norcholan, das $C_{22}H_{38}$ als Bisnorcholan bezeichnet wird. So wurde Trioxy-norcholen (Kazuno 1943) (*6*),

Tabelle I

Tioxy-bufosterocholensäure.		Trioxy-isosterocholensäure.		Tetraoxynorbufostan.	
Triacetoxy-bufosterocholansäure-methylester.	Fp. 132°	Triacetoxy-isosterocholansäure-methylester.	Fp. 102°	—	
Trioxy-bufosterocholansäure-methylester.	Fp. 170° $[\alpha]_D = +26.69°$	Trioxy-isosterocholansäure-methylester.	Fp. 162° $[\alpha]_D = +33.78°$	—	
Tetraoxy-bufostan.	Fp. 216°	Tetraoxy-isobufostan.	Fp. 215°	Tetraoxynorbufostan.	Fp. 238°
Triketo-bufosterocholansäure.	Fp. 260°	Triketo-isosterocholansäure.	Fp. 234°	Triketo-norbufosterocholansäure.	Fp. 221-222°

Tabelle II

Cholsäure	Dosoxycholsäure	Hyodesoxycholsäure	Ursodesoxycholsäure	Chenodesoxycholsäure	Lithocholsäure
3, 7, 12-Trioxycholanol-24 Fp. 225°	3,12-Dioxycholanol-24 Fp. 165°	3,6-Dioxycholanol-24 Fp. 169°	α-3,β-7-Dioxycholanol-24 Fp. 113°	α-3,α-7-Dioxycholanol-24	3-Oxycholanol-24 Fp. 177°

Trioxy-cholan von der Formel $C_{24}H_{42}O_3$ (Kazuno (1940) (*7*) aus der Krötengalle und Tetraoxycholan (Kazuno u. Kurauti 1934) (*8*) aus der Galle von *Rana Catesbina* Shaw vorgefunden.

BESCHREIBUNG DER VERSUCHE

I. Trioxy-bufosterocholensäurederivate

Triacetoxy-bufosterocholensäure-methylester—1 g. des Esters von Fp. 172° wurde in wasserfreiem Pyridin gelöst, mit 8 ml. von frisch destilliertem Essigsäure-anhydrid versetzt und 30 Stunden lang auf dem Wasserbade erwärmt. Die Reaktionslösung wurde in viel Wasser eingegossen, die dabei abgeschiedene Fällung in Aether aufgenommen und der Aetherauszug nach dem Waschen erst mit verdünnter Salzsäure, dann mit Wasser verdampft. Der Rückstand wurde aus Methanol-Wasser umkrystallisiert. 83～5°.

3.292, 3.585 mg. Subst. : 8.405, 9.120 mg. CO_2, 2.590, 2.860 mg. H_2O.
$C_{35}H_{54}O_8$ Ber. C 69.72 H 9.34
Gef. C 69.63, 69.38 H 8.80, 8.93

Triacetoxy-bufosterocholansäure-methylester.—0.5 g. des Ester vom Schmelzpt. 85° wurde in 10 ml. Eissessig gelöst, mit 0.1 g. Platinoxyd nach Adams versetzt und unter Leitung von Wasserstoff geschüttelt. Die Reaktionslösung wurde nach dem Filtrieren von Platinoxyd in viel Wasser eingegossen, die dabei abgeschiedene Fällung aus Methanol-Wasser umkrystallisiert. Tafeln vom Schmelzpt. 132°.

3.555, 3.490 mg. Subst. : 9.055, 8.870 mg. CO_2, 2.935, 2.850 mg. H_2O.
$C_{35}H_{56}O_8$ Ber. C 69.49 H 9.33
Gef. C 69.47, 69.31 H 9.24, 9.14

Trioxy-bufosterocholensäure-methylester (*Fp. 172°*)—spec. Drehung : 0.0766 g. Subst., in 10 ml. Alkohol, $l=2$ dm., $\alpha=-0.48°$ $[\alpha]_D=-31.33°$

Trioxy-bufosterocholansäure-methylester—Der Methylester vom Schmelzpt. 172° wurde in Alkohol üblicherweise mit Platinoxyd nach Adams unter Wasserstoffgas katalytisch hydriert. Die durch Eingiessen der Reaktionslösung in viel Wasser abgeschiedene Fällung wurde aus Methanol-Wasser umkrystallisiert. Nadeln vom Schmelzpt. 169°～170°.

spec. Drehung : 0.0487 g. Subst. in 10 ml. Methanol.
$l=1$ dm., $\alpha=+0.13°$ $[\alpha]_D=+26.69°$
4.150 mg. Subst. : 10.730 mg. CO_2, 4.020 mg. H_2O
$C_{29}H_{50}O_5 \cdot H_2O$ Ber. C 70.10 H 10.56
Gef. C 70.51 H 10.84

Trioxy-bufosterocholensäure-äthylester.—Der Aethylester wurde üblicherweise mittelst Aethanols und Schwefelsäure aus Trioxy-bufosterocholensäure hergestellt. Der Ester wurde aus Methanol umkrystallisiert. Tafeln vom Schmelzpt 153°.

3.750 mg. Subst. : 9.762 mg. CO_2, 3.430 mg. H_2O
$C_{30}H_{50}O_5 \cdot H_2O$ Ber. C 70.81 H 10.31
Gef. C 71.00 H 10.23

Trioxy-bufosterocholenol-28—1.1 g. Trioxy-bufosterocholensäure-aethylester wurde in 80 ml. absolutem Alkohol gelöst, mit 7.5 g. Natrium allmählich stückweise versetzt, auf dem Ölbade bei 180° anderthalbstunden lang erhitzt, mit 50 ml. Wasser versetzt und auf dem Wasserbade eine Stunde lang erwärmt. Das Reaktionsgemisch wurde in viel Wasser eingegossen, die dabei abgeschiedene neutrale Fällung in Essigaether aufgenommen und nach dem Waschen mit Wasser und Trocknen mit Pottasche eingeengt. Der Rückstand wurde aus Essigaether umkrystallisiert. Prismen vom Schmelzpt. 216°. Der Krystall addiert Brom und entfärbt die Kameleonlösung. Die Substanz ist also ungesättigt. Bei vorsichtiger Oxydation des Trioxy-bufosterocholenol-28 wird Triketo-bufosterocholensäure vom Schmelzpt. 269° geliefert.

3.900 mg. Subst. : 10.690 mg. CO_2, 3.750 mg. H_2O
$C_{28}H_{48}O_4$ Ber. C 74.95 H 10.79
Gef. C 74.76 H 10.76

Trioxy-bufosterocholanol-28—0.2 g. Trioxy-bufosterocholenol-28 wurde in 5 ml. Eisessig gelöst, mit 50 mg. Platinoxyd nach Adams versetzt, unter Leitung von Wasserstoff katalytisch hydriert, wobei nach einigen Minuten ein Mol Wasserstoff aufgenommen wurde, und weiter nach 40 Minuten vom Platinoxyd abfiltriert. Das Filtrat wurde mit Sodalösung neutralisiert und die dabei abgeschiedene Fällung abfiltriert. Diese Fällung wurde mit alkoholischer Kalilaugelösung 30 Minuten hydrolysiert und das Hydrolysat unter Zusatz von Wasser von Alkohol verdampft. Die dabei abgeschiedene Fällung wurde aus Alkohol-Wasser umkrystallisiert. Die Substanz addiert nicht mehr Brom und entfärbt nicht mehr die Kameleonlösung. Tafeln vom Schmelzpt. 216°.

3.470 mg. Subst. : 9.535 mg. CO_2, 3.470 mg. H_2O
$C_{28}H_{50}O_4$ Ber. C 74.61 H 11.19
Gef. C 74.94 H 11.19

II. Trioxy-isosterocholensäurederivate

Triacetoxy-isosterocholensäure-methylester—1 g. Trioxy-isosterocholensäure-methylester vom Schmelzpt. 220° wurde in 15 ml. wasserfreiem Pyridin gelöst, mit 10 ml. eines frisch destillierten Essigsäureanhydrids versetzt und auf dem Wasserbade 28 Stunden erwärmt. Das Reaktionsgemisch wurde in viel Wasser eingegossen, die dabei abgeschiedene Fällung in Aether aufgenommen und der Aetherauszug nach dem Waschen erst mit verdünter Salzsäure und dann mit Wasser abgedampt. Die dabei abgeschiedene Fällung wurde aus Methanol-Wasser umkrystallisiert. Der Krystall schmilzt bei 104°.

3.500, 3.700 mg. Subst. : 8.850, 9.330 mg. CO_2 2.770, 2.885 mg. H_2O

$C_{35}H_{54}O_8 \cdot H_2O$ Ber. C 68.65 H 9.09
Gef. C 68.96, 68.77 H 8.85, 8.72

Triacetoxy-isosterocholansäure-methylester—0.5 g. Acetat wurde in 5 ml. Eisessig gelöst, mit 0.1 g. Platinoxyd versetzt und unter Leitung von Wasserstoff katalytisch hydriert. Der auf diese Weise erhaltene Triacetoxy-isosterocholansäure-methylester wurde aus Methanol-Wasser umkrystallisiert. Prismen vom Schmelzpt. 102°. Diese Substanz addiert nicht Brom und entfärbt nicht die Kameleonlösung.

3.711 mg. Subst. : 9.444 mg. CO_2, 3.057 mg. H_2O.
$C_{35}H_{56}O_8$ Ber. C 69.49 H 9.33
Gef. C 69.41 H 9.22

Trioxy-isosterocholensäure-methylester von Fp. 220°—spec. Drehung : 0.0685 g. Subst. in 10 ml. Alkohol, l = 1 dm.,

$\alpha = +0.38^\circ$, $[\alpha]_D = +55.47^\circ$

Trioxy-isosterocholansäure-methylester—Der Ester wurde in üblicher Weise durch katalytische Hydrierung aus Trioxy-isosterocholensäure-methylester hergestellt. Der aus Methanol-Wasser umkrystallisierte Ester krystallisiert in Nadeln, sintert bei 90° und schmilzt bei 162°.

spec. Drehung : 0.0444 g. Subst., in 10 ml. Methanol, l = 1 dm.
$\alpha = +0.15^\circ$, $[\alpha]_D = +33.78^\circ$
4.000 mg. Subst. : 10.030 mg. 3.765 mg. H_2O.
$C_{29}H_{50}O_5 \cdot H_2O$ Ber. C 68.18 H 10.66
Gef. C 68.39 H 10.53

Trioxy-isosterocholensäure-äthylester—0.5 g. Trioxy-isosterocholensäure wurde in 50 ml. absolutem Alkohol gelöst und unter vorsichtigem Zusatz von 2.5 g. konzentrierter Schwefelsäure auf dem Wasserbade gekocht. Die durch Eingiessen der Reaktionslösung in Wasser abgeschiedene Fällung wurde aus Methanol-Wasser umkrystallisiert. Nadeln vom Schmelzpt. 173°. Die Substanz addiert Brom und entfärbt die Kameleonlösung.

4.340 mg. Subst. : 11.310 mg. CO_2, 3.880 mg. H_2O.
$C_{30}H_{50}O_5 \cdot H_2O$ Ber. C 70.81 H 10.31
Gef. C 71.07 H 10.00

Trioxy-isosterocholenol-25—0.3 g. Trioxy-isosterocholensäure-aethylester wurde in 50 ml. absolutem Alkohol mit 5 g. Natrium auf dem Ölbad bis auf 165° reduziert und dann unter Zusatz von 50 ml. Wasser 2 Stunden lang hydrolysiert, das Hydrolysat in Wasser abgegossen und von Alkohol abgedampft. Die dabei abgeschiedene neutrale Fällung wurde in Essigaether aufgenommen und der Essigaetherauszug nach dem Waschen mit Wasser und Trocknen mit Pottasche eingeengt. Die dabei erhaltene Fällung wurde aus Essigaether umkrystallisiert. Prismen vom Schmelzpt. 220°. Die Substanz addiert Brom und entfärbt die Kameleonlösung. Die Substanz liefert bei vorsichtiger Oxydation mit Chromsäure eine Triketo-isosterocholensäure vom Schmelzpt. 241°, die sich mit der durch Oxydation der Trioxy-isosterocholensäure erhaltenen Säure identisch erweist.

3.770 mg. Subst. : 10.320 mg. CO_2, 3.500 mg. H_2O

$C_{28}H_{48}O_4$	Ber.	C 74.95	H 10.79
	Gef.	C 74.66	H 10.39

Trioxy-isosterocholanol-25—50 mg. Trioxy-isosterocholenol-25 wurden in 5 ml. Eisessig mit 10 mg. Platinoxyd und Wasserstoff katalytisch hydriert. Die von Platinoxyd abfiltrierte, durch Eingiessen der Reaktionslösung abgeschiedene Fällung wurde mit methylalkoholischer Kalilaugelösung 30 Minuten lang hydrolysiert. Das Hydrolysat wurde durch Zusatz von Wasser und dann durch Abdampfen von Alkohol befreit und die dabei abgeschiedene Fällung aus Alkohol-Wasser umkrystallisiert. Tafeln vom Schmelzpt. 215°. Die Substanz addiert nicht Brom und entfärbt nicht die Kameleonlösung.

3.125 mg. Substanz : 8.563 mg. CO_2, 3.070 mg. H_2O.

$C_{28}H_{50}O_4$	Ber.	C 74.61	H 11.19
	Gef.	C 74.74	H 10.99

Bei der Mischprobe zeigt Trioxy-bufosterocholanol-28 (Fp 216°) mit dem Trioxy-isosterocholanol-25 (Fp 215°) eine Schmelzpunktdepression und zwar sintert bei 208° und schmilzt bei 213°.

III. Cholanol-24-Derivate

3,7,12-Trioxy-cholanol-24—4 g. Cholsäureaethylester wurden in 100 ml. absolutem Alkohol gelöst, erwärmend bei 120°∼180° auf dem Ölbade mit 5 g. Natrium versetzt und eine Stunde lang reduziert. Die Reaktionslösung wurde in Wasser eingegossen und die dabei abgeschiedene Fällung mit einer 5 prozentigen alkoholischen Kalilaugelösung eine Stunde lang hydrolysiert. Der neutrale Teil des Hydrolysats wurde in Essigaether aufgenommen, der Essigaetherauszug nach dem Trocknen abgedampft und der Rückstand aus Alkohol-Wasser umkrystallisiert. Spindeln vom Schmelzpunkt 225°. Die Substanz zeigt eine Hammarstensche Reaktion.

3.210 mg. Subst. : 8.600 mg. CO_2, 3.020 mg. H_2O.

$C_{24}H_{42}O_4$	Ber.	C 73.04	H 10.74
	Gef.	C 73.07	H 10.53

3, 12-Dioxycholanol-24—5 g. Desoxycholsäureaethylester wurden in 100 ml. absolutem Alkohol gelöst, unter Erwärmung bei 175° mit 10 g. Natrium versetzt und eine Stunde lang reduziert. Von dem Reduktionsprodukte wurde nach der Hydrolyse mit alkoholischer Kalilauge der neutrale Anteil in Essigaether aufgenommen und die aus dem Essigaetherauszug erhaltene Substanz aus Essigaether umkrystallisiert. Prismatischer Krystall vom Schmelzpunkt 165°. Der Krystall sintert bei 153° und schmilzt bei 165°. Dieser Krystall wurde aus absolutem Alkohol umkrystallisiert. Der hierbei erhaltene Krystll sintert bei 156° und schmilzt bei 185°.

2.860 mg. Subst. : 7.990 mg. CO_2, 2.910 mg. H_2O.
$C_{24}H_{42}O_3$ Ber. C 76.12 H 11.19
Gef. C 76.19 H 11.38

Die aus absolutem Alkohol umkrystallisierte Substanz vom Schmelzpunkt 186° wurde nochmals aus Alkohol-Wasser umkrystallisiert. Die hier erhaltene Substanz krystallisiert in Prismen, sintert bei 93° und schmilzt bei 105°

4.610, 4.120 mg. Subst. : 11.700, 10.475 mg. CO_2, 4.562, 4.100 mg. H_2O
$C_{24}H_{42}O_3$ Ber. C 69.51 H 11.19
Gef. C 69.22, 69.34 H 11.07 11.13

Die Substanz, die aus Alkohol-Wasser umkrystallisiert wurde, wurde nochmal aus Eisessig umkrystallisiert. Die hier erhaltenen grossen Nadeln sintern bei 93° und schmelzen bei 105°, genau wie es bei der Umkrystallisation aus Alkohol-Wasser der Fall war.

3.490 mg, Subst. : 8.900 mg. CO_2, 3.470 mg. H_2O
$C_{24}H_{42}O_3 \cdot 2H_2O$ Ber. C 69.51 H 11.19
Gef. C 69.55 H 11.12

Aus den oben erhaltenen Ergebnissen geht hervor, dass für die Bildung der Choleinsäure aus Desoxycholsäure nicht nur die sekundären Alkoholgruppen in der Stellung an C_3 und C_{12}, von denen die eine an C_3 α-ständig ist, sondern auch die Carboxylgruppe des Desoxycholsäuremoleküls notwenidg sind.

3, 6-Dioxycholanol-24—0.5 g. Hyodesoxycholsäureaethylester wurde in 80 ml. absolutem Alkohol gelöst und unter Zusatz von 5 g. Natrium in gleicher Weise wie oben reduziert. Der aus dem Reduktionsprodukte durch Hydrolyse erhaltene neutrale Anteil wurde aus Essigaether umkrystallisiert. Tafeln vom Schmelzpunkt 168°~169°.

3.525 mg. Subst. : 9.890 mg. CO_2, 3.570 mg. H_2O
$C_{24}H_{42}O_3$ Ber. C 76.12 H 11.19
Gef. C 76.52 H 11.33

3,7-Dioxycholanol-24—

1) Aus Ursodesoxycholsäure :

2 g. Ursodesoxycholsäureaethylester wurden in 50 ml. absolutem Alkohol gelöst und unter Zusatz von 5 g. Natrium erwärmend bei 160° reduziert. Der aus dem Reduktionsgemisch durch Hydrolyse erhaltene neutrale Anteil wurde aus allen möglichen Lösungsmitteln umkrystallisiert, aber nicht krystallinisch sondern amorph erhalten. Die Substanz schmilzt bei 113°.

2) Aus Chenodesoxycholsäure :

3, 7-Dioxycholanol aus Chenodesoxycholsäure wurde nicht krystallinisch sondern immer als Öl erhalten.

3-Oxycholanol-24—Die durch Behandlung der Desoxycholsäure mit Acetylchlorid erhaltene 3-Acetoxy-12-oxycholansäure wurde in üblicher Weise mit Chromsäure oxydiert. Die hier erhaltene 3-Acetoxy-12-ketocholansäure wurde in Semicarbazon vom Schmelzpunkt 210° übergeführt. 2.5 g. Semicarbazon wurden in 25 ml. absolutem Alkohol gelöst und mit 2 g. Natrium versetzt. Diese Lösung wurde in einem Jenaer Glasrohr 6 Stunden lang erhitzt. Aus diesem Reaktionsgemisch wurde durch Behandlung in üblicher Weise 1 g. Lithocholsäure erhalten. Lithocholsaäureethylester.

3.460 mg. Subst. : 9.780 mg. CO_2, 3.380 mg. H_2O

$C_{26}H_{44}O_3$	Ber.	C 77.17	H 10.96
	Gef.	C 77.09	H 10.93

Der Lithocholsäure-aethylester vom Schmelzpunkt 97° wurde in Alkohol mit Natrium üblicherweise reduziert. Der aus dem Reduktionsprodukte erhaltene Alkohol wurde aus Alkohol-Wasser oder aus Essigaether umkrystallisiert. Nadeln vom Schmelzpunkt 177°.

3-Oxycholanol-24—

3.675 mg. Subst. : 10.690 mg. CO_2, 3.760 mg. H_2O

$C_{24}H_{42}O_2$	Ber.	C 79.46	H 11.68
	Gef.	C 79.33	H 11.45

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LETTERS TO THE EDITORS

A BIOCHEMICAL CHLORINATION IN STREPTOMYCES

Sirs:

The biochemical chlorination in the biogenesis of chlortetracycline was studied as its one typical case in *Streptomyces.*

(1) The biochemical chlorination in the biosynthesis of chlortetracycline is inhibited by the presence of bromine ion (0.80 per cent. as NaBr) in the culture medium.

(2) Under the existence of bromine ion, the chlortetracycline producing organism gives a deschloro-antibiotic (antibiotic -X) having the tetracycline nucleus.

(3) The molecular structure of antibiotic -X was later decided as tetracycline itself (*1*) in co-operation with H. Ogawa *et al.*, division of organic chemistry in our laboratory. In 1940, P. W. Clutterbuck *et al.* (*2*) reported that bromine ion might be substitutive for chlorine ion in *Caldariomyces Fumago* which produced an organic chlorine containing metabolite, caldariomycin. But according to the evidences described above and the survey of bromine ion during the shaking culture, bromine ion is not substitutive for chlorine ion as in this case of *Streptomyces.*

(4) Although bromine ion exists in the culture medium (0.40 per cent. to 3.20 per cent. as NaBr), chlortetracycline is recognized as the by-product in the cultured broth. (T/C. T- ratio 80/20 to 90/10 generally, 95/5 as maximum case by paperchromatographic determination.) The production of chlortetracycline under the existence of bromine ion is defined as " the residual reaction ".

(5) It is not yet attained to obtain the biochemical mutant, which does not go the residual reaction, from the many monospore-cultures treated with various mutagenic agents. This seems to support the suggestion that at least double mutant may be required to obtain the mutant which does not go the residual reaction.

(6) Among the 18 organic compounds, 4 compounds: 2-thiouracil, thiourea, 2-mercapto-benzothiazol and thionalide inhibit the residual reaction markedly or completely. (About 1,500 γ/ml. as tetracycline monohydrochloride is produced at T/C. T-ratio 99/1 under the existence of 0.80 per cent. NaBr plus 0.002 per cent. 2-mercapto-benzothiazol in

the culture medium which initially contains 0.04 per cent. NaCl derived from the natural substances as the ingredients.) Among the above 4 effective compounds, 2-mercapto-benzothiazol completely inhibits the biochemical chlorination without the existence of bromine ion. On the other hand, 2-thiouracil, thiourea and 2-mercapto-benzothiazol are known as the antithyroid substances (*3*), and 2-thiouracil and thiourea are well known as the inhibitors for the copper enzymes such as tyrosinase and ascorbase (*4*). And the all above 4 effective compounds are able to chelate with copper ion (*5*). These evidences suggest that the biochemical chlorination in this organism is the interesting bio-phenomenon related to the biochemical iodination in thyroid gland of mammalia and also copper may participate in the mechanism of the biochemical chlorination in this organism. But the mechanism of the participation of copper in this biochemical chlorination is obscure. In 1953, B. Weiss (*6*) reported that copper ion was the indispensable enzymatic requirement for the utilization of iodide by the cell free preparation of beef thyroid tissue and the oxidation of I^- to I might link with the reduction of enzymatic Cu^{++}. The analogy from these evidences obtained by B. Weiss (*6*), may permit the suggestion that copper plays a vital role as the enzymatic requirement in the mechanism of the biochemical chlorination in *Streptomyces*.

Details on the above studies will be reported in the Journal of Japanese Biochemical Society.

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